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(12) United States Patent
Middendorf et al.

(10) **Patent No.:** **US 6,207,421 B1**
(45) **Date of Patent:** **Mar. 27, 2001**

(54) **DNA SEQUENCING AND DNA TERMINATORS**

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(73) Assignee: **Li-Cor, Inc.**, Lincoln, NE (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/505,072**

(22) Filed: **Jul. 21, 1995**

Related U.S. Application Data

(60) Continuation-in-part of application No. 08/275,232, filed on Jul. 14, 1994, now abandoned, which is a division of application No. 07/950,734, filed on Sep. 24, 1989, now Pat. No. 5,346,603, which is a continuation of application No. 07/799,712, filed on Nov. 6, 1991, now abandoned, which is a continuation of application No. 07/632,605, filed on Dec. 24, 1990, now abandoned, which is a continuation of application No. 07/078,279, filed on Jul. 27, 1987, now abandoned, which is a division of application No. 06/594,676, filed on Mar. 29, 1984, now Pat. No. 4,729,947, which is a continuation-in-part of application No. 08/288,461, filed on Aug. 10, 1994, now Pat. No. 5,534,125, which is a division of application No. 08/018,806, filed on Feb. 17, 1993, now Pat. No. 5,360,523, which is a division of application No. 07/763,230, filed on Sep. 20, 1991, now Pat. No. 5,230,781, and a division of application No. 07/570,503, filed on Aug. 21, 1990, now Pat. No. 5,207,880, which is a continuation-in-part of application No. 07/078,279, filed on Jul. 27, 1987, which is a division of application No. 06/594,676, filed on Mar. 29, 1984, now Pat. No. 4,729,947, and a continuation-in-part of application No. 08/204,627, filed on Mar. 1, 1994, now Pat. No. 5,571,388, which is a continuation-in-part of application No. 07/860,140, filed on Mar. 30, 1992, now Pat. No. 5,366,603, which is a division of application No. 07/763,230, filed on Sep. 20, 1991, now Pat. No. 5,230,781, which is a continuation-in-part of application No. 07/570,503, filed on Aug. 21, 1990, now Pat. No. 5,207,880, which is a continuation-in-part of application No. 07/078,279, filed on Jul. 27, 1987, which is a division of application No. 06/594,676, filed on Mar. 29, 1984, now Pat. No. 4,729,947.

(51) **Int. Cl.**^{7} **C12Q 1/68**; C12P 19/34; C07H 19/00; C07H 21/00

(52) **U.S. Cl.** **435/91.1**; 435/6; 435/91.2; 536/22.1; 536/25.3; 536/25.32

(58) **Field of Search** 435/6, 91.1, 91.2; 536/22.1, 25.3, 25.32

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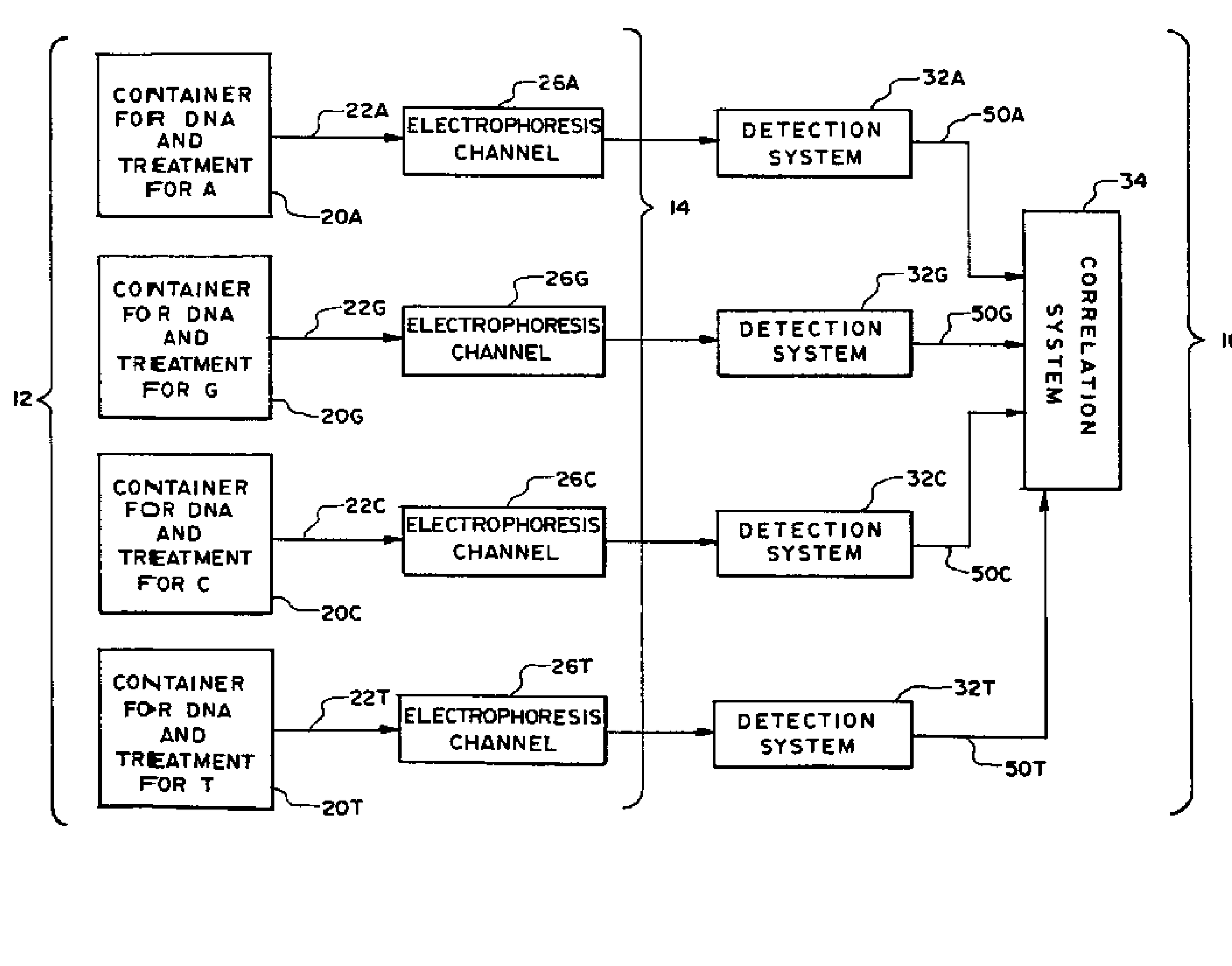
(57) **ABSTRACT**

A universal terminator includes a heterocycle other than naturally occuring DNA heterocycles such as adenine, cytosine, guanine and thymine. In terminating strand synthesis, the hybridized primer-template is split into four aliquots. In the "A" vial is added DNA polymerase and normal amounts of "C", "G", and "T" deoxynucleotides, along with a reduced amount of "A" deoxynucleotide and an amount of the universal terminator such that statistically the universal terminator has less than a one percent chance of being incorporated at sites where a "C", "G", or "T" deoxynucleotide should be incorporated and about a one percent chance of being incorporated at sites where an "A" deoxynucleotide should be incorporated. Similar strategies are followed for the "C", "G", and "T" vials, except that the amount of "C", "G" and "T" deoxynucleotides are reduced for their respective vial.

3 Claims, 13 Drawing Sheets



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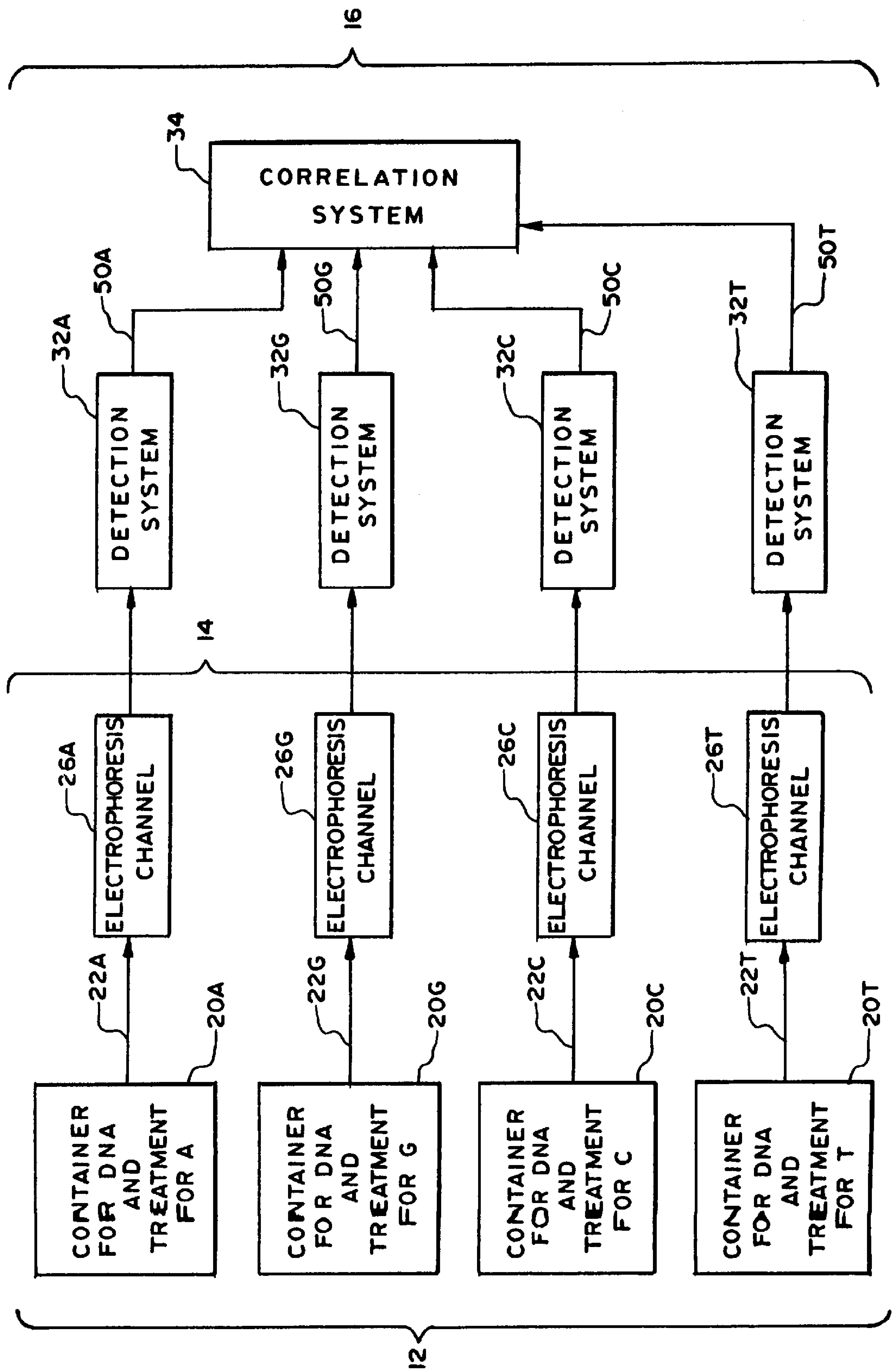
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34
CORRELATION SYSTEM
50A
50G
50C
50T
32A
32G
32C
32T
DETECTION SYSTEM
DETECTION SYSTEM
DETECTION SYSTEM
DETECTION SYSTEM
14
26A
26G
26C
26T
ELECTROPHORESIS CHANNEL
ELECTROPHORESIS CHANNEL
ELECTROPHORESIS CHANNEL
ELECTROPHORESIS CHANNEL
22A
22G
22C
22T
20A
20G
20C
20T
CONTAINER FOR DNA AND TREATMENT FOR A
CONTAINER FOR DNA AND TREATMENT FOR G
CONTAINER FOR DNA AND TREATMENT FOR C
CONTAINER FOR DNA AND TREATMENT FOR T
12

FIG. 1

FIG. 3

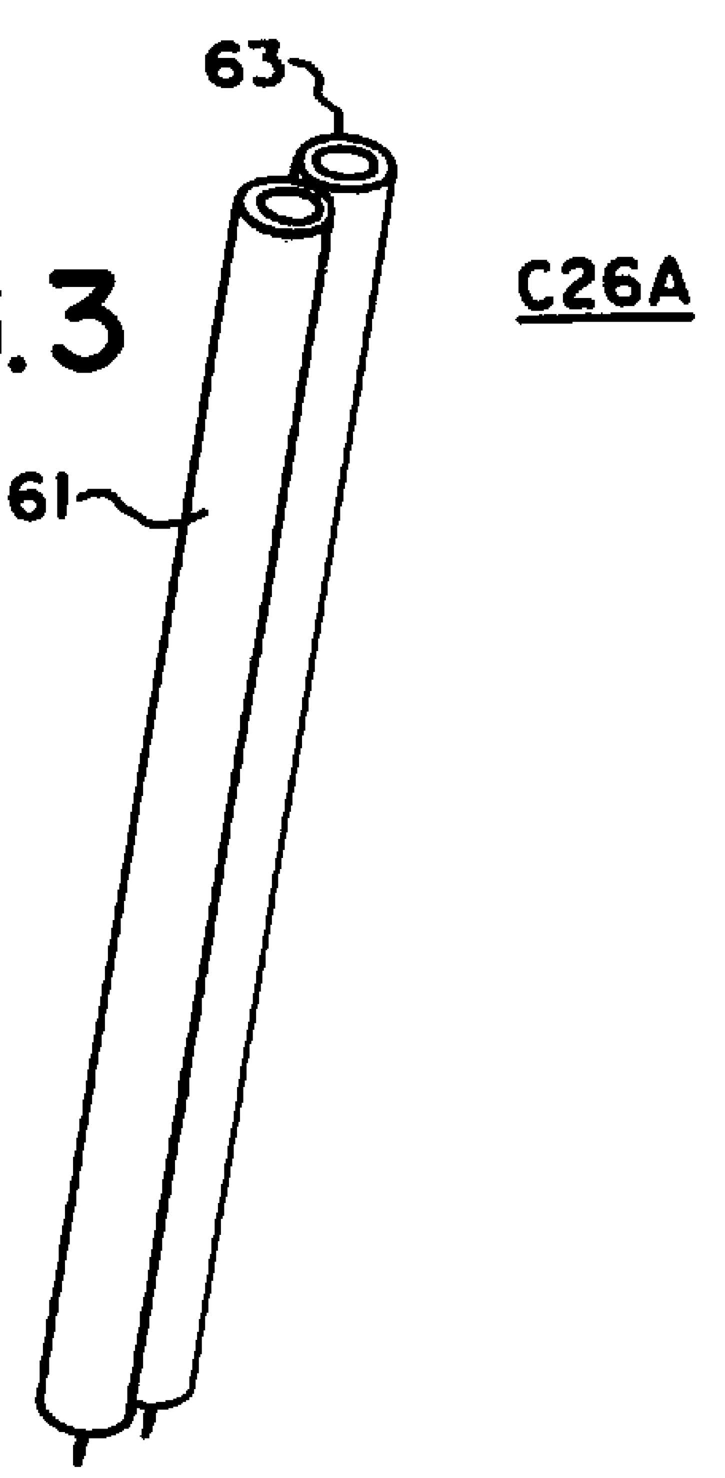
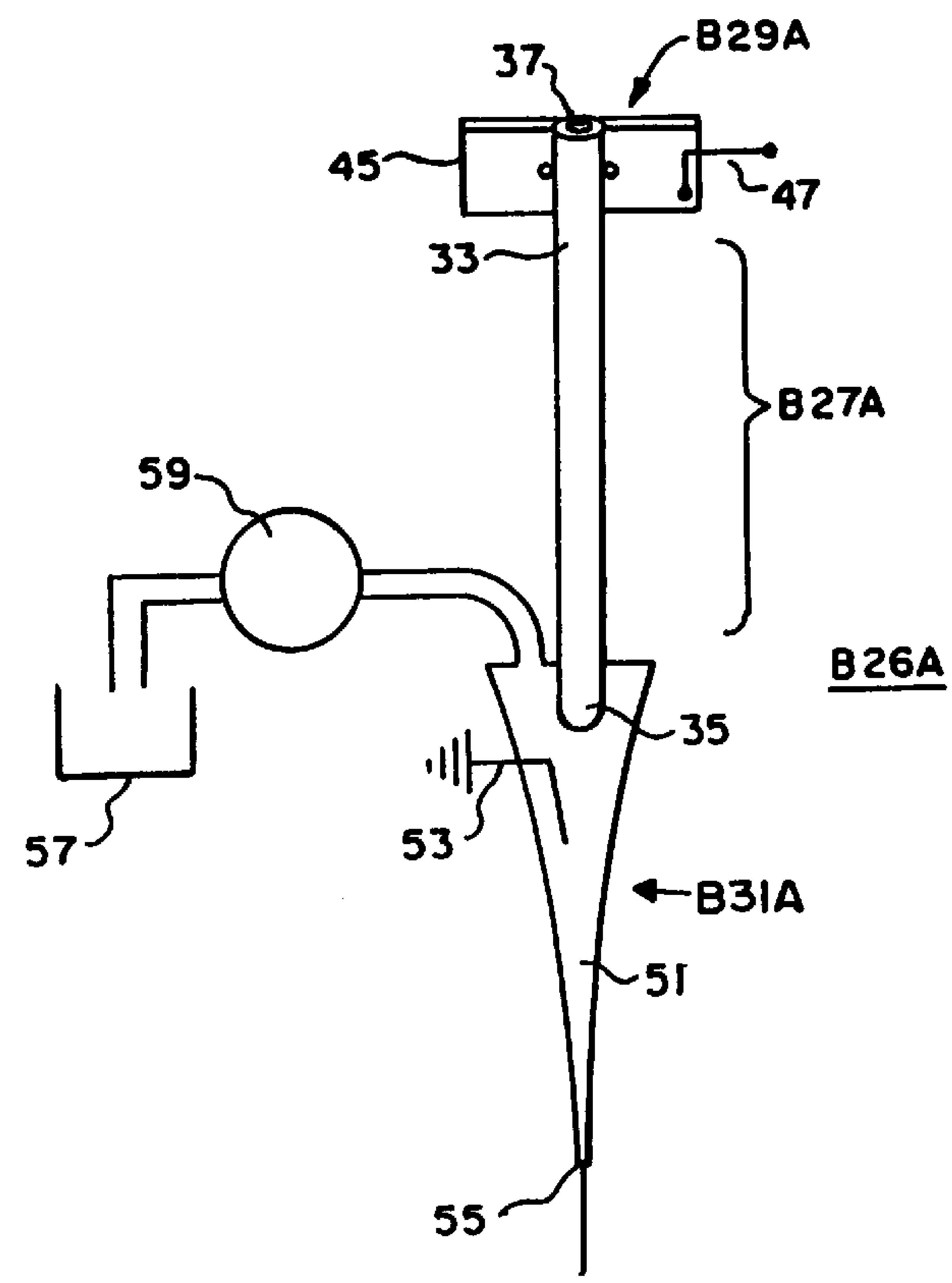


FIG. 2



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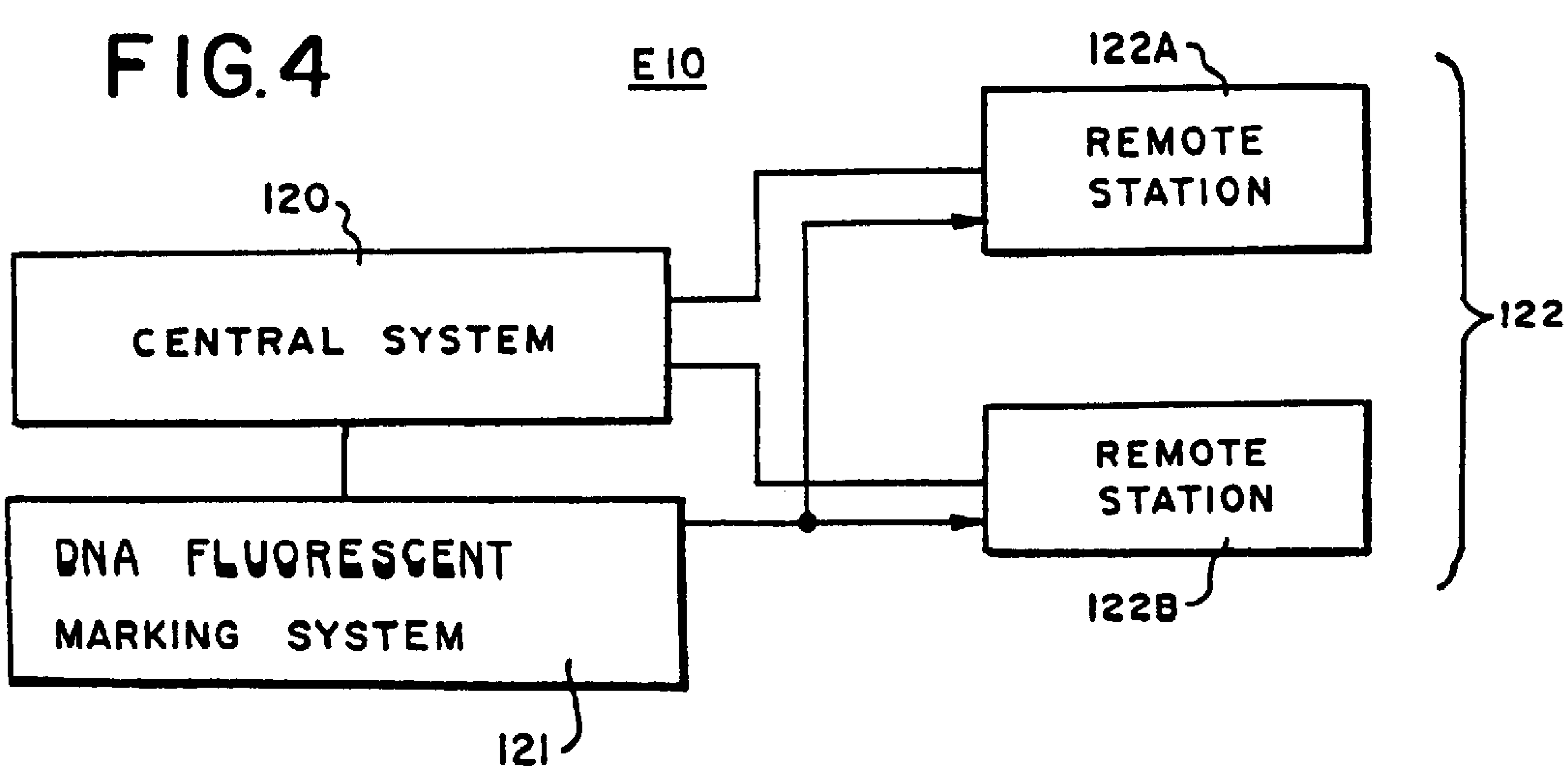


FIG. 5

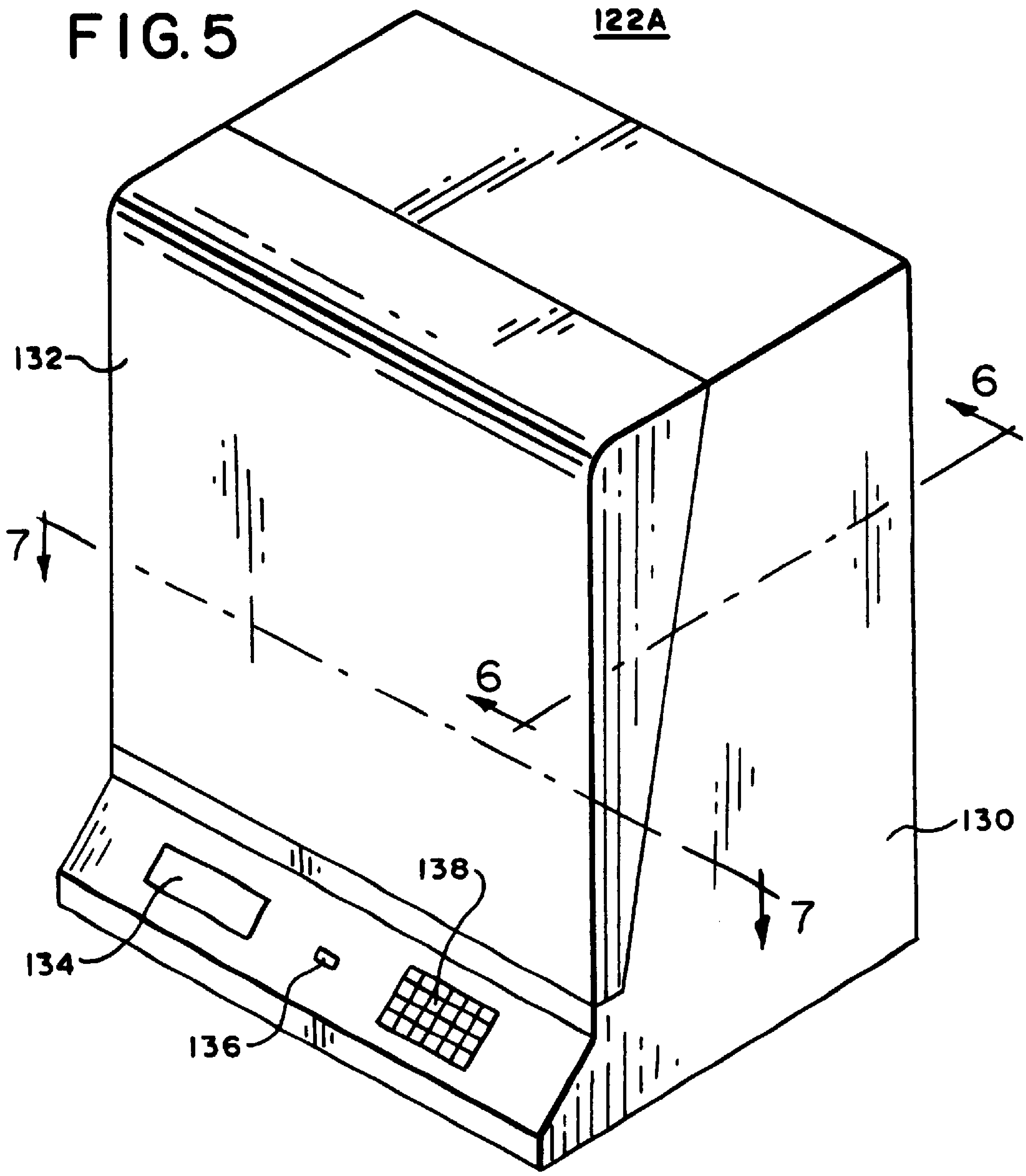
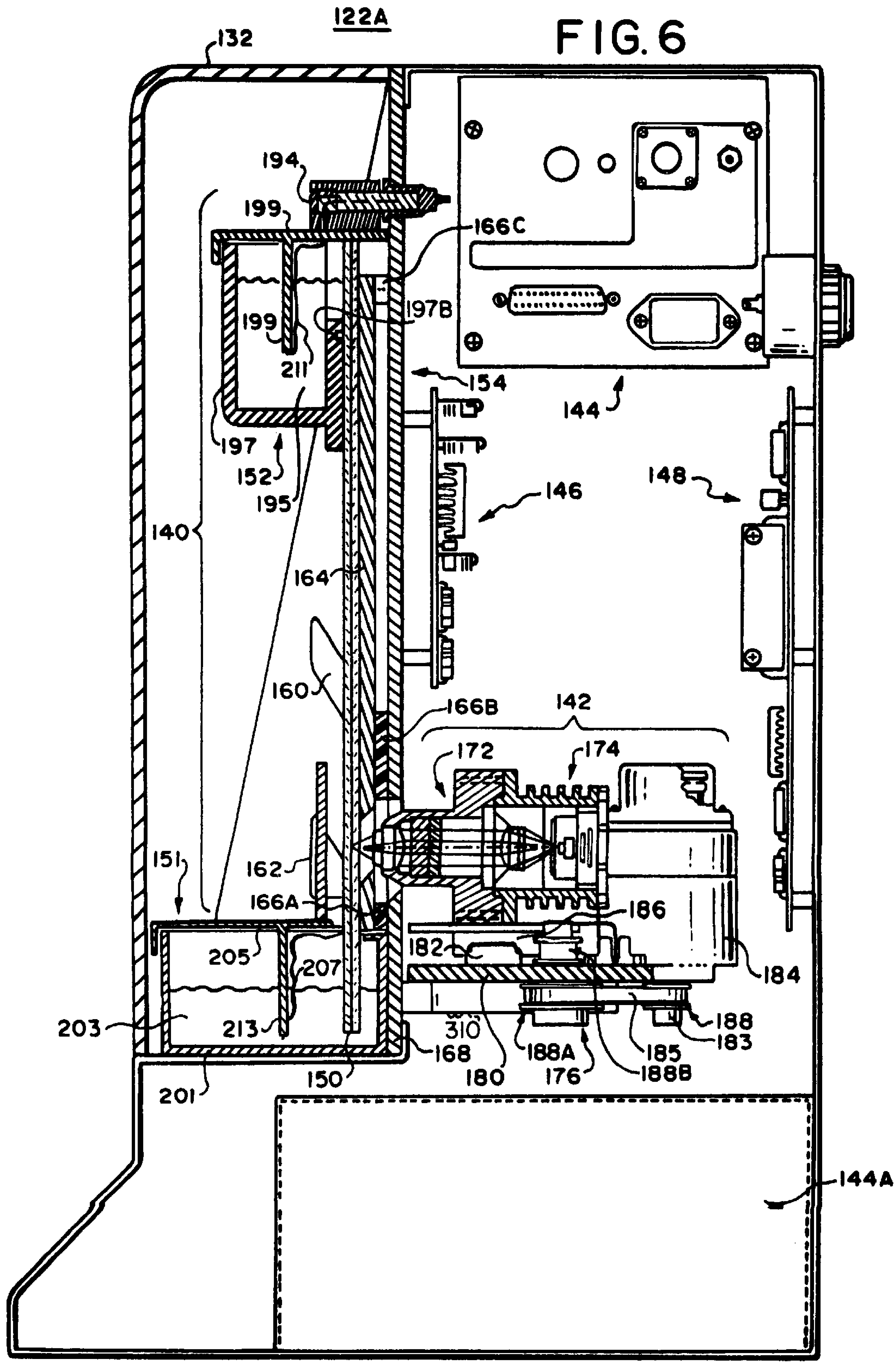


FIG. 6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194
199
166C
197B
199
211
154
144
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195
146
148
140
164
160
166B
142
172
174
151
162
166A
186
182
184
205
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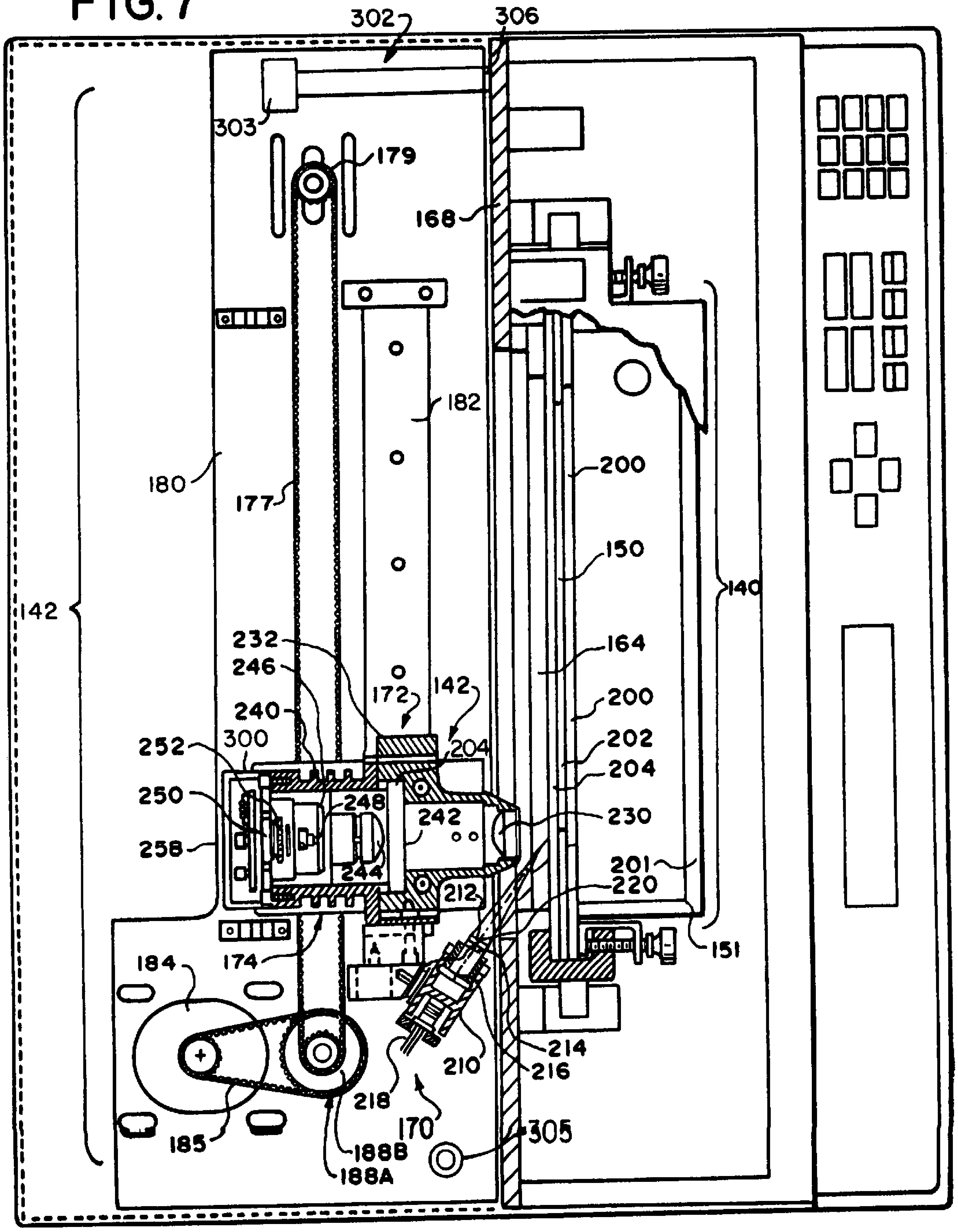
213
310
188
183
168
188A
185
150
180
176
188B
201
144A

FIG. 7



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306
303
179
168
182
180
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200
150
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164
232
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252
300
204
248
250
242
230
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184
214
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218
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185
188B
188A

FIG. 8

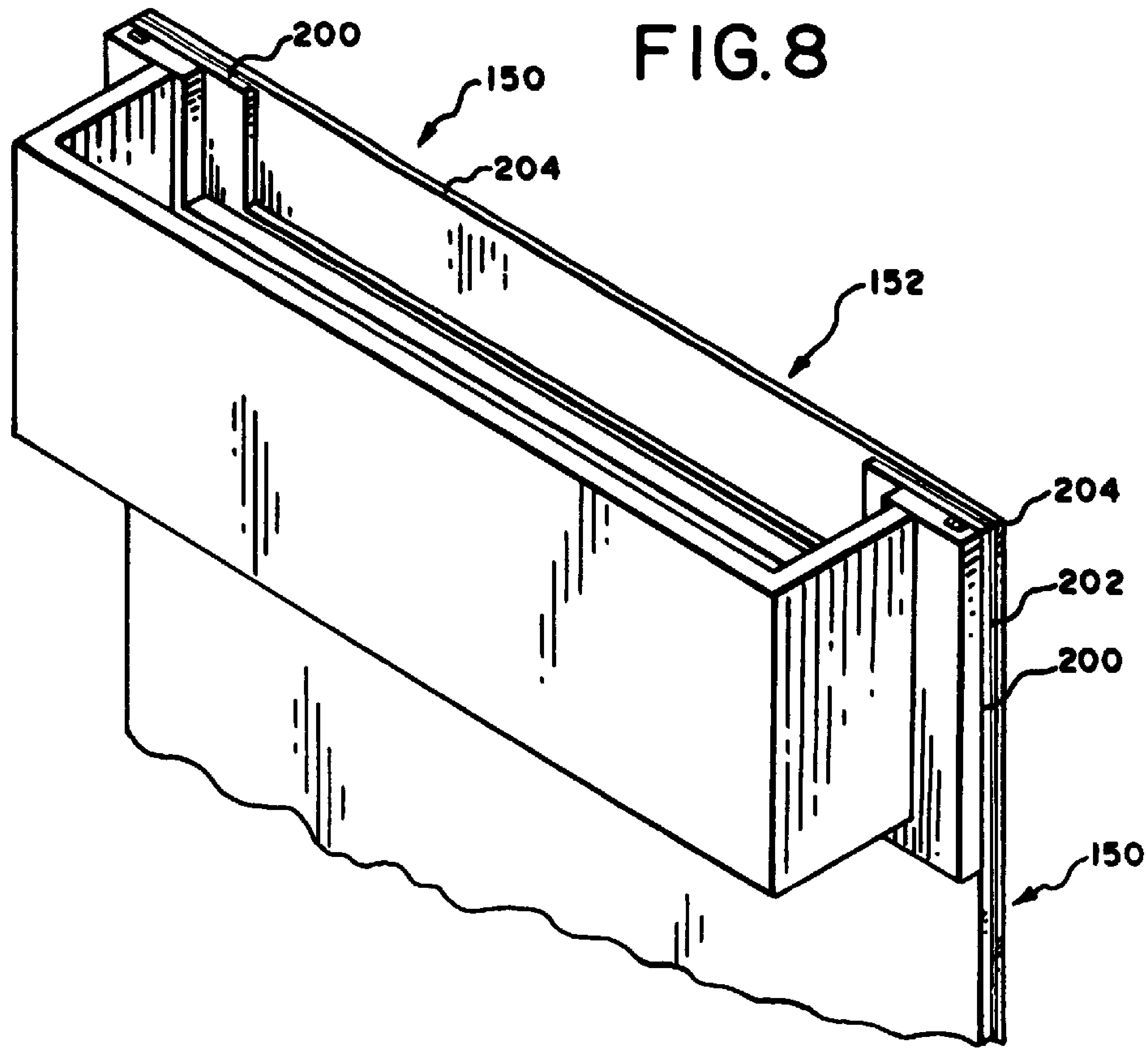
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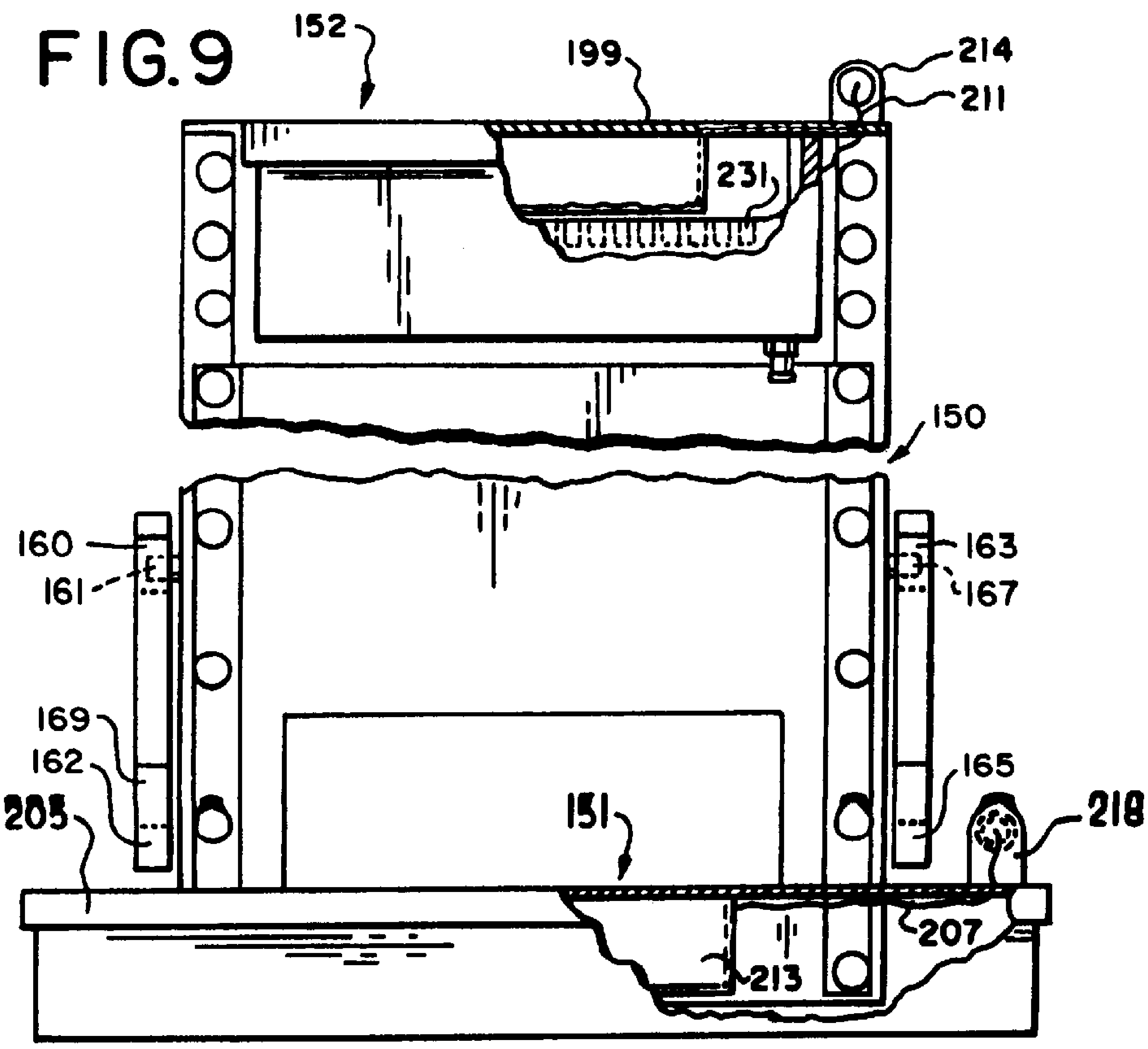
FIG. 9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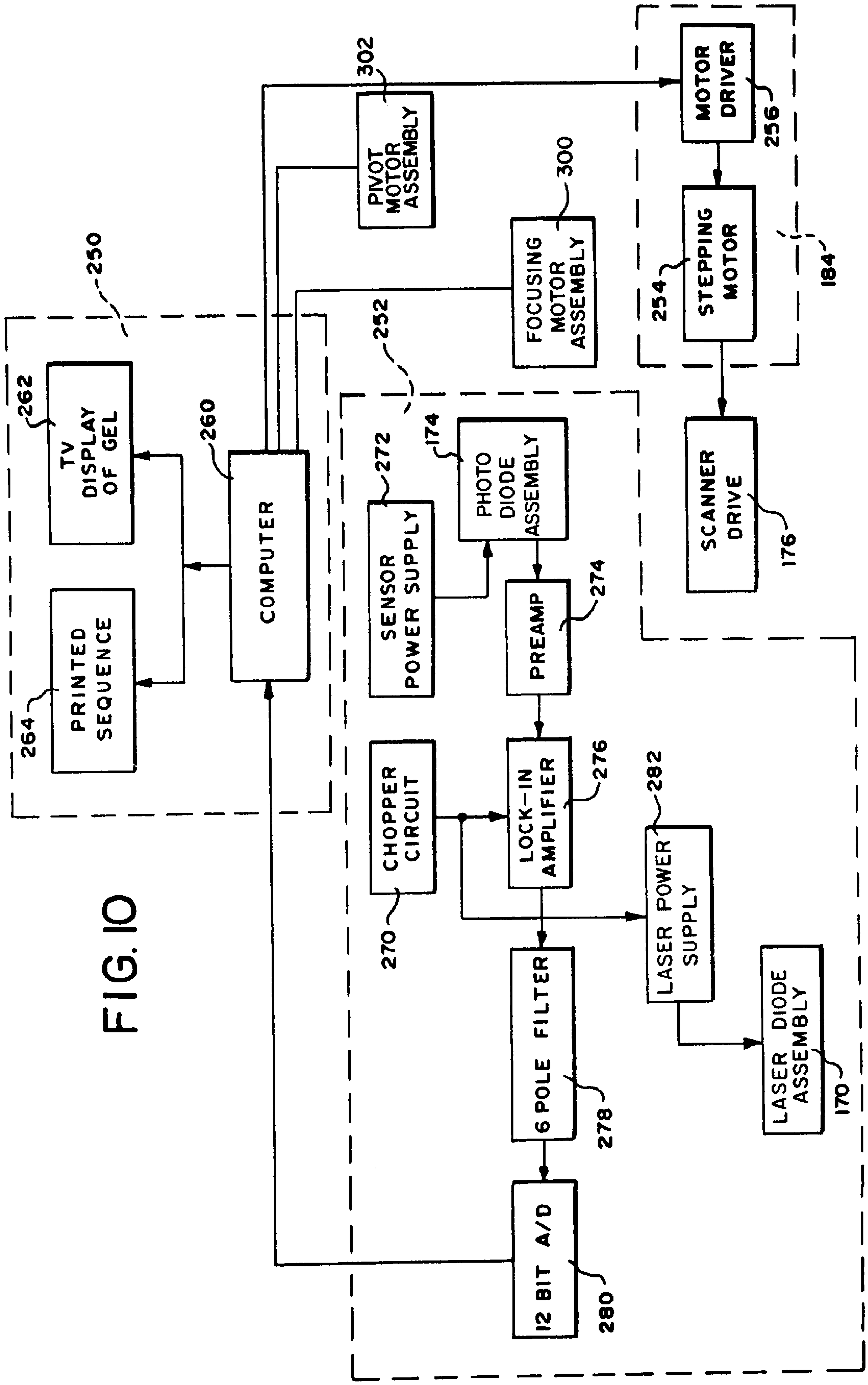
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TV DISPLAY OF GEL
262
PRINTED SEQUENCE
264
COMPUTER
260
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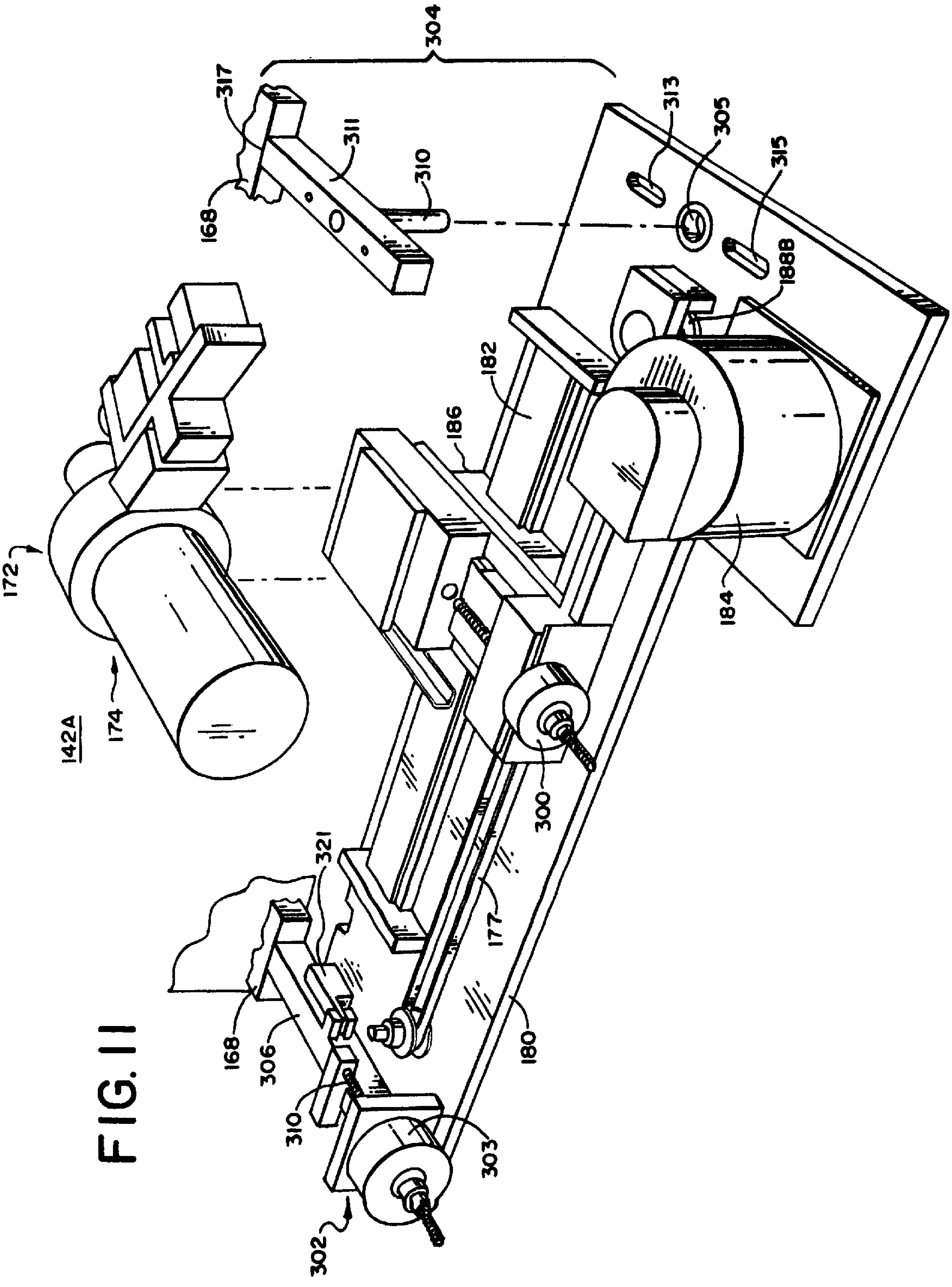
PIVOT MOTOR ASSEMBLY
300
FOCUSING MOTOR ASSEMBLY
MOTOR DRIVER
256
STEPPING MOTOR
254
184
252
PHOTO DIODE ASSEMBLY
174
SENSOR POWER SUPPLY
272
PREAMP
274
SCANNER DRIVE
176
CHOPPER CIRCUIT
270
LOCK-IN AMPLIFIER
276
LASER POWER SUPPLY
282
6 POLE FILTER
278
LASER DIODE ASSEMBLY
170
12 BIT A/D
280

FIG. 10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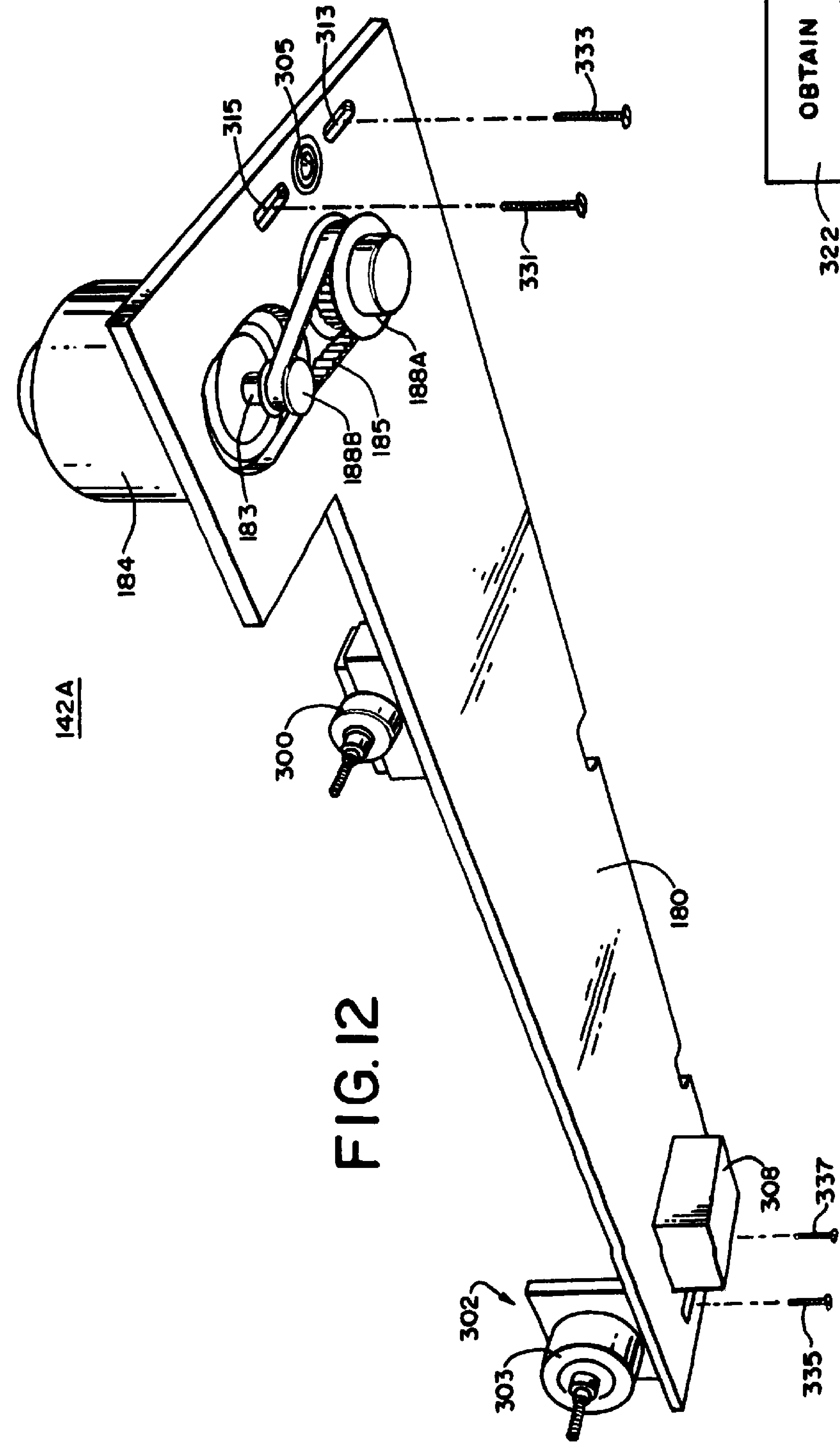
180

FIG. 11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184
183
188B
185
188A
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305
313
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180
308
337
302
303
335

FIG.12

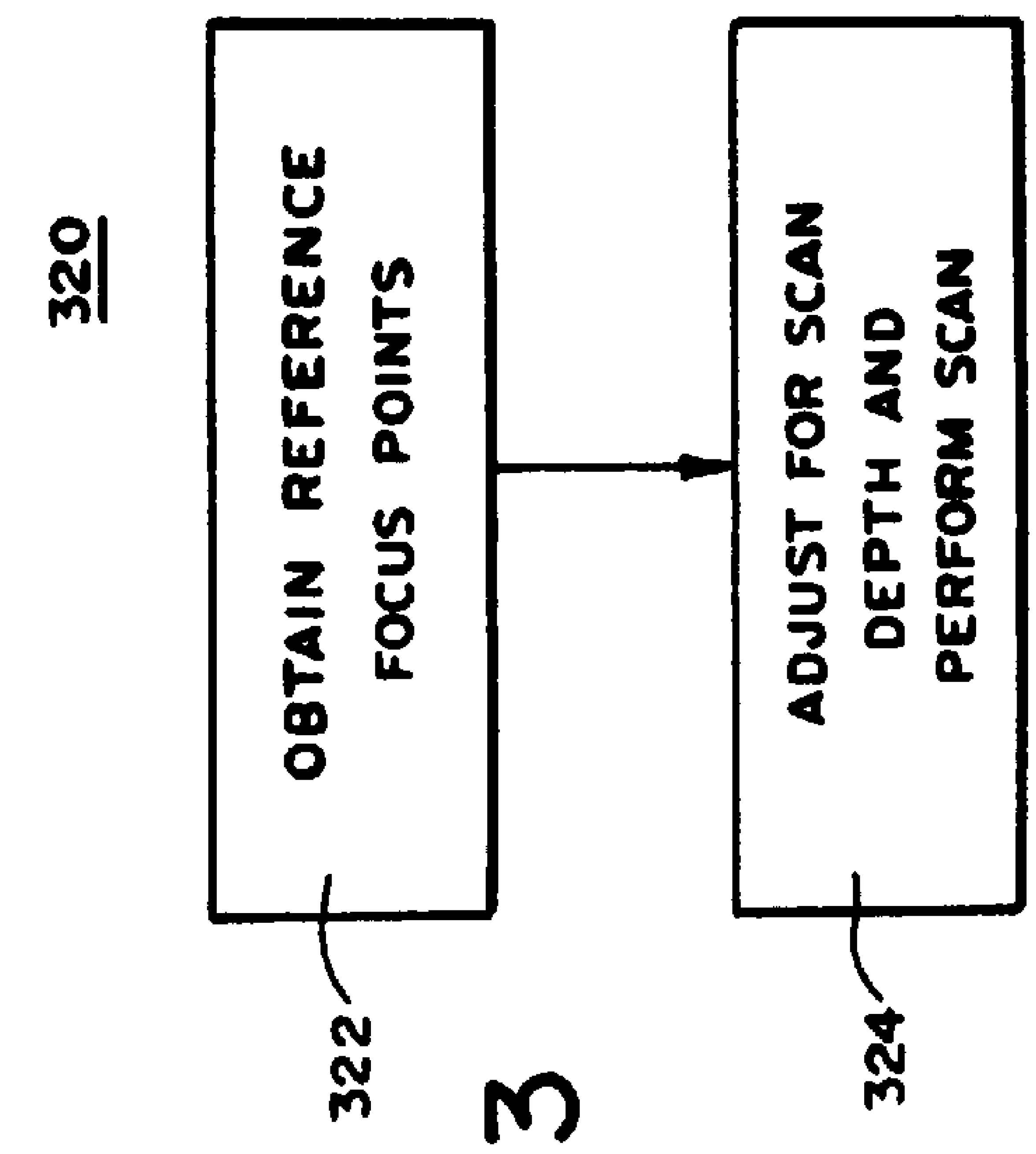
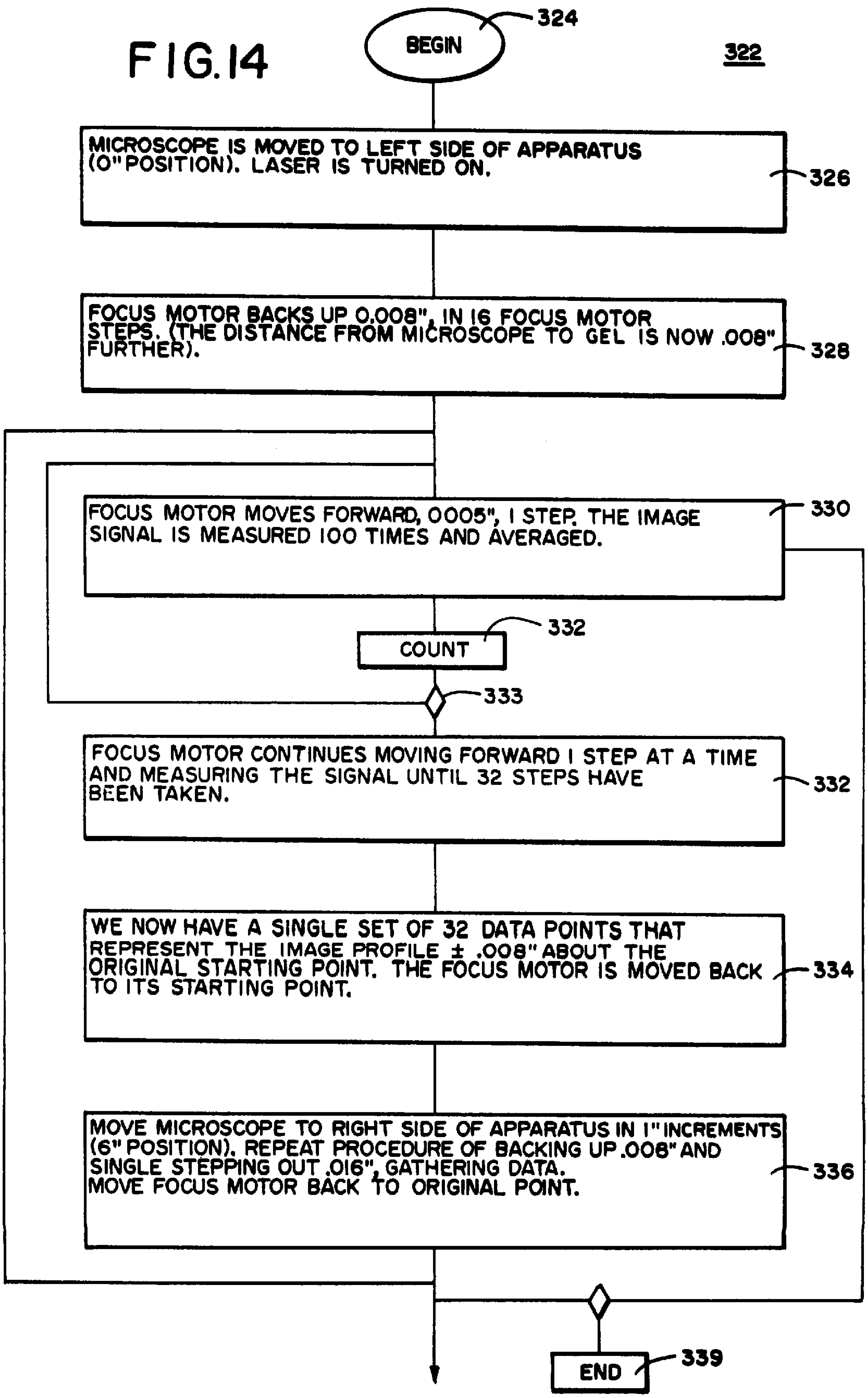

320
OBTAIN REFERENCE FOCUS POINTS
ADJUST FOR SCAN DEPTH AND PERFORM SCAN
322
324

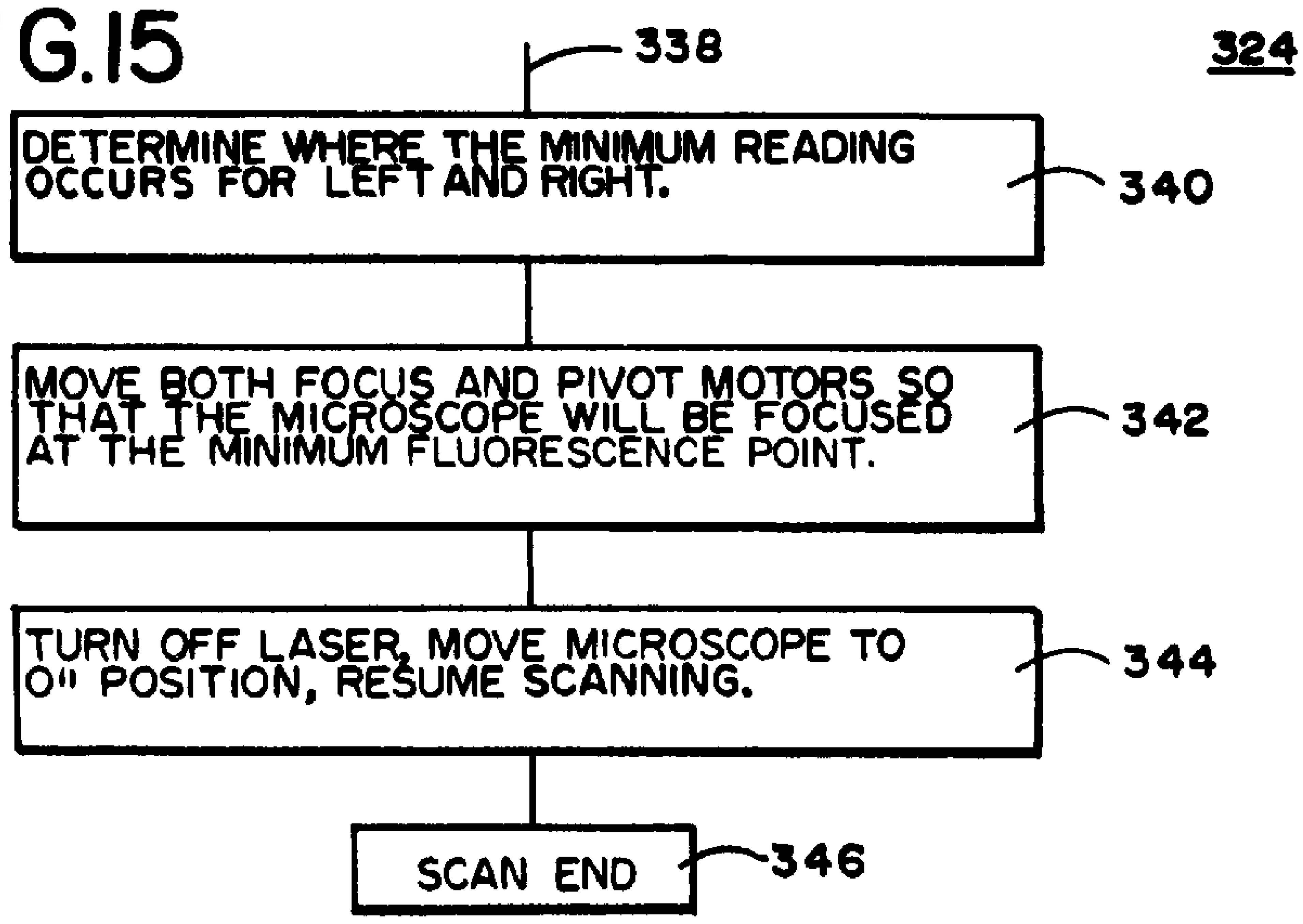
FIG.13

FIG. 14



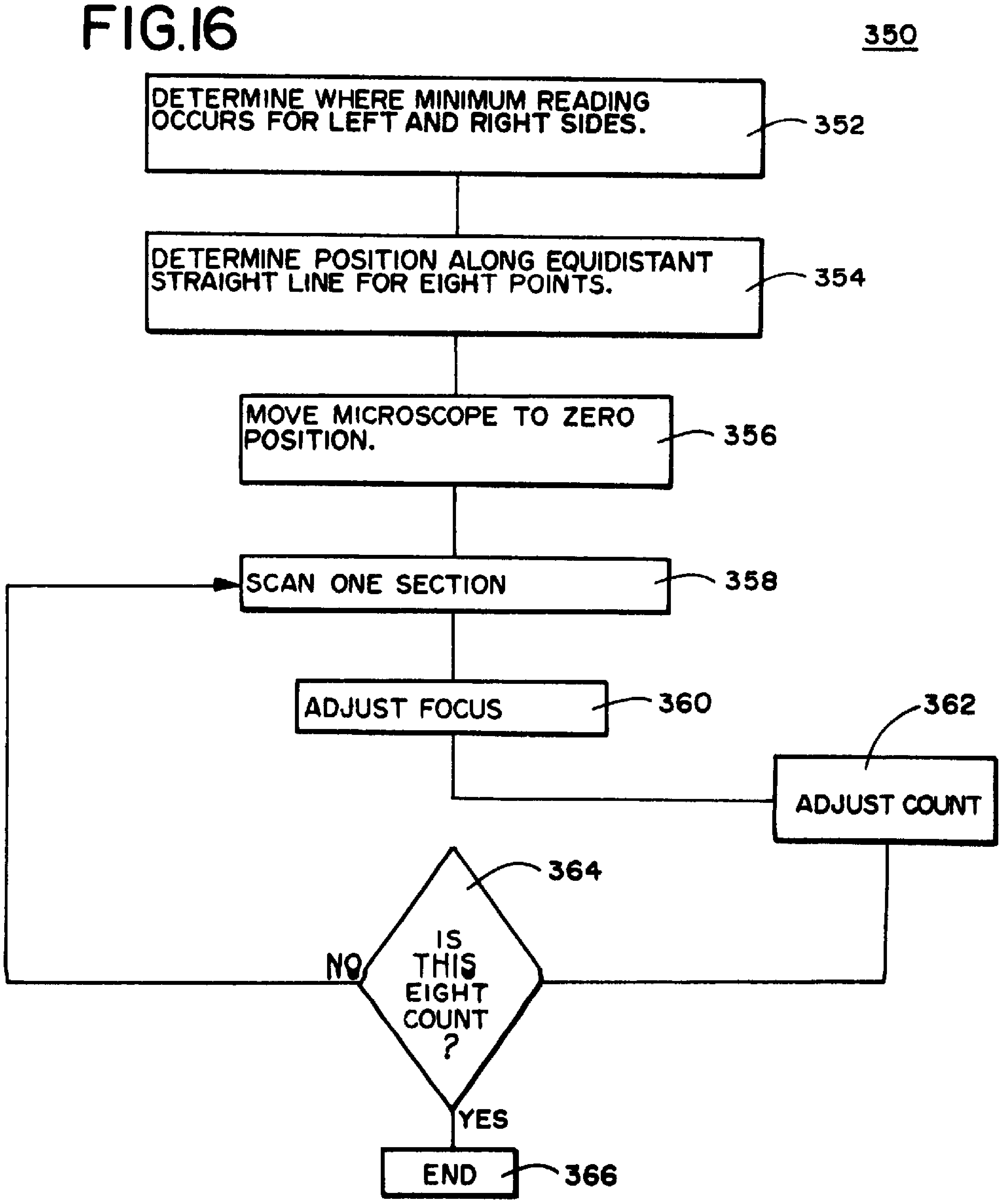
BEGIN
324
322
MICROSCOPE IS MOVED TO LEFT SIDE OF APPARATUS (0" POSITION). LASER IS TURNED ON.
326
FOCUS MOTOR BACKS UP 0.008", IN 16 FOCUS MOTOR STEPS. (THE DISTANCE FROM MICROSCOPE TO GEL IS NOW .008" FURTHER).
328
FOCUS MOTOR MOVES FORWARD, 0005", 1 STEP. THE IMAGE SIGNAL IS MEASURED 100 TIMES AND AVERAGED.
330
COUNT
332
333
FOCUS MOTOR CONTINUES MOVING FORWARD 1 STEP AT A TIME AND MEASURING THE SIGNAL UNTIL 32 STEPS HAVE BEEN TAKEN.
332
WE NOW HAVE A SINGLE SET OF 32 DATA POINTS THAT REPRESENT THE IMAGE PROFILE ± .008" ABOUT THE ORIGINAL STARTING POINT. THE FOCUS MOTOR IS MOVED BACK TO ITS STARTING POINT.
334
MOVE MICROSCOPE TO RIGHT SIDE OF APPARATUS IN 1" INCREMENTS (6" POSITION). REPEAT PROCEDURE OF BACKING UP .008" AND SINGLE STEPPING OUT .016", GATHERING DATA. MOVE FOCUS MOTOR BACK TO ORIGINAL POINT.
336
END
339

FIG.15



338
324
DETERMINE WHERE THE MINIMUM READING OCCURS FOR LEFT AND RIGHT.
340
MOVE BOTH FOCUS AND PIVOT MOTORS SO THAT THE MICROSCOPE WILL BE FOCUSED AT THE MINIMUM FLUORESCENCE POINT.
342
TURN OFF LASER, MOVE MICROSCOPE TO 0" POSITION, RESUME SCANNING.
344
SCAN END
346

FIG.16



350
DETERMINE WHERE MINIMUM READING OCCURS FOR LEFT AND RIGHT SIDES.
352
DETERMINE POSITION ALONG EQUIDISTANT STRAIGHT LINE FOR EIGHT POINTS.
354
MOVE MICROSCOPE TO ZERO POSITION.
356
SCAN ONE SECTION
358
ADJUST FOCUS
360
362
ADJUST COUNT
364
IS THIS EIGHT COUNT ?
NO
YES
END
366

FIG.17

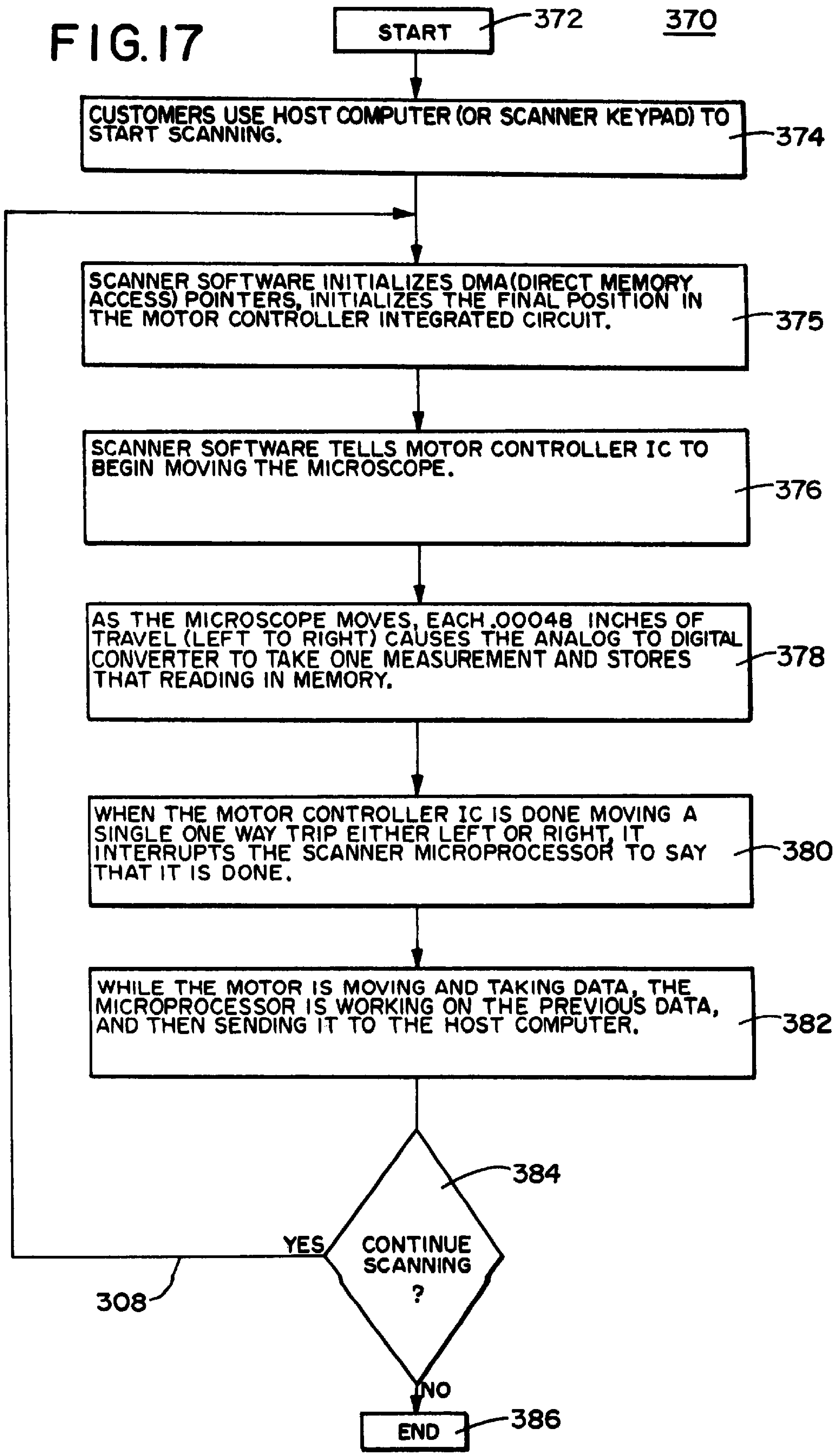

START
372
370
CUSTOMERS USE HOST COMPUTER (OR SCANNER KEYPAD) TO START SCANNING.
374
SCANNER SOFTWARE INITIALIZES DMA(DIRECT MEMORY ACCESS) POINTERS, INITIALIZES THE FINAL POSITION IN THE MOTOR CONTROLLER INTEGRATED CIRCUIT.
375
SCANNER SOFTWARE TELLS MOTOR CONTROLLER IC TO BEGIN MOVING THE MICROSCOPE.
376
AS THE MICROSCOPE MOVES, EACH .00048 INCHES OF TRAVEL (LEFT TO RIGHT) CAUSES THE ANALOG TO DIGITAL CONVERTER TO TAKE ONE MEASUREMENT AND STORES THAT READING IN MEMORY.
378
WHEN THE MOTOR CONTROLLER IC IS DONE MOVING A SINGLE ONE WAY TRIP EITHER LEFT OR RIGHT, IT INTERRUPTS THE SCANNER MICROPROCESSOR TO SAY THAT IT IS DONE.
380
WHILE THE MOTOR IS MOVING AND TAKING DATA, THE MICROPROCESSOR IS WORKING ON THE PREVIOUS DATA, AND THEN SENDING IT TO THE HOST COMPUTER.
382
384
YES
CONTINUE SCANNING ?
308
NO
END
386

FIG. 18

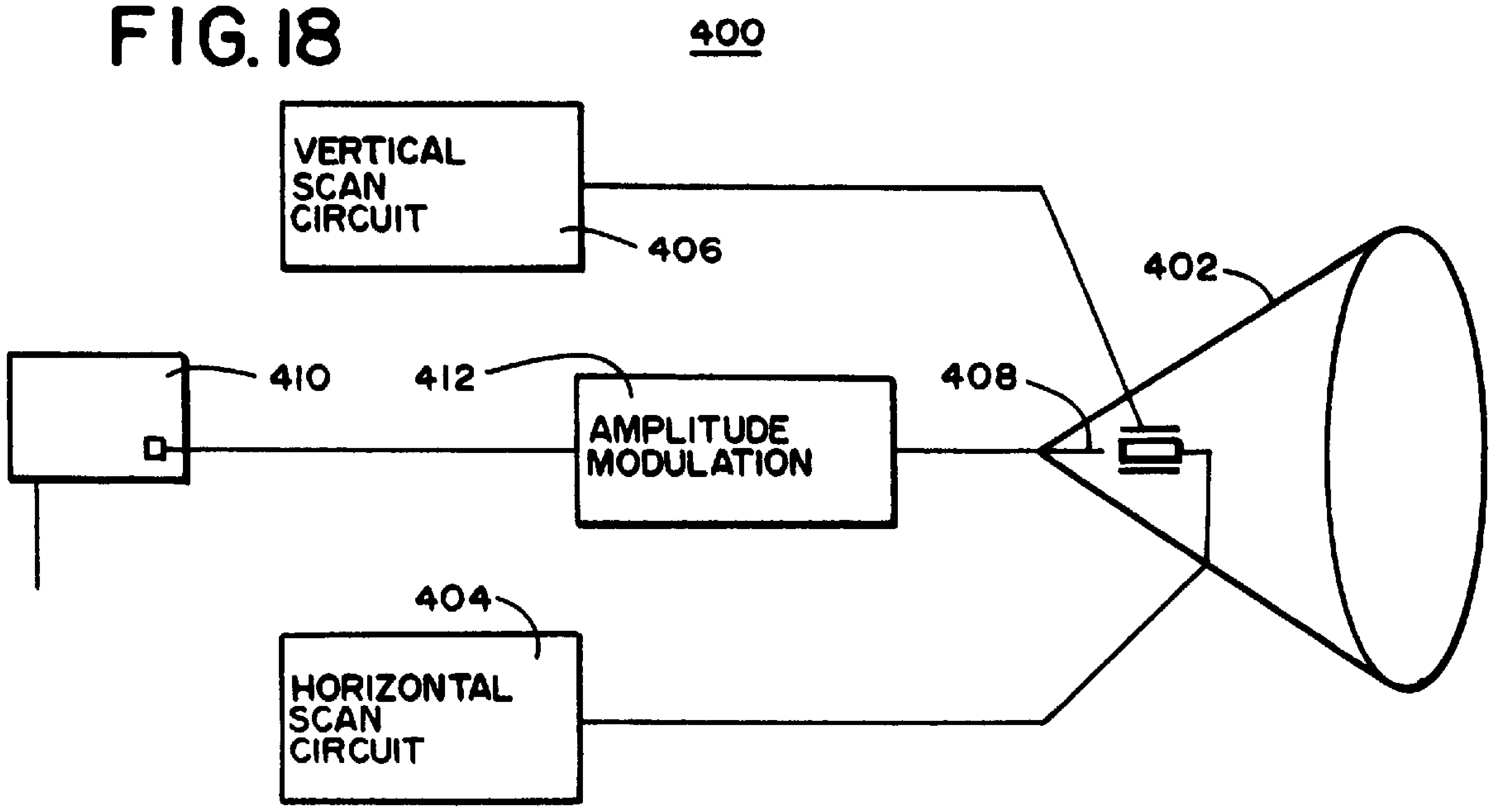
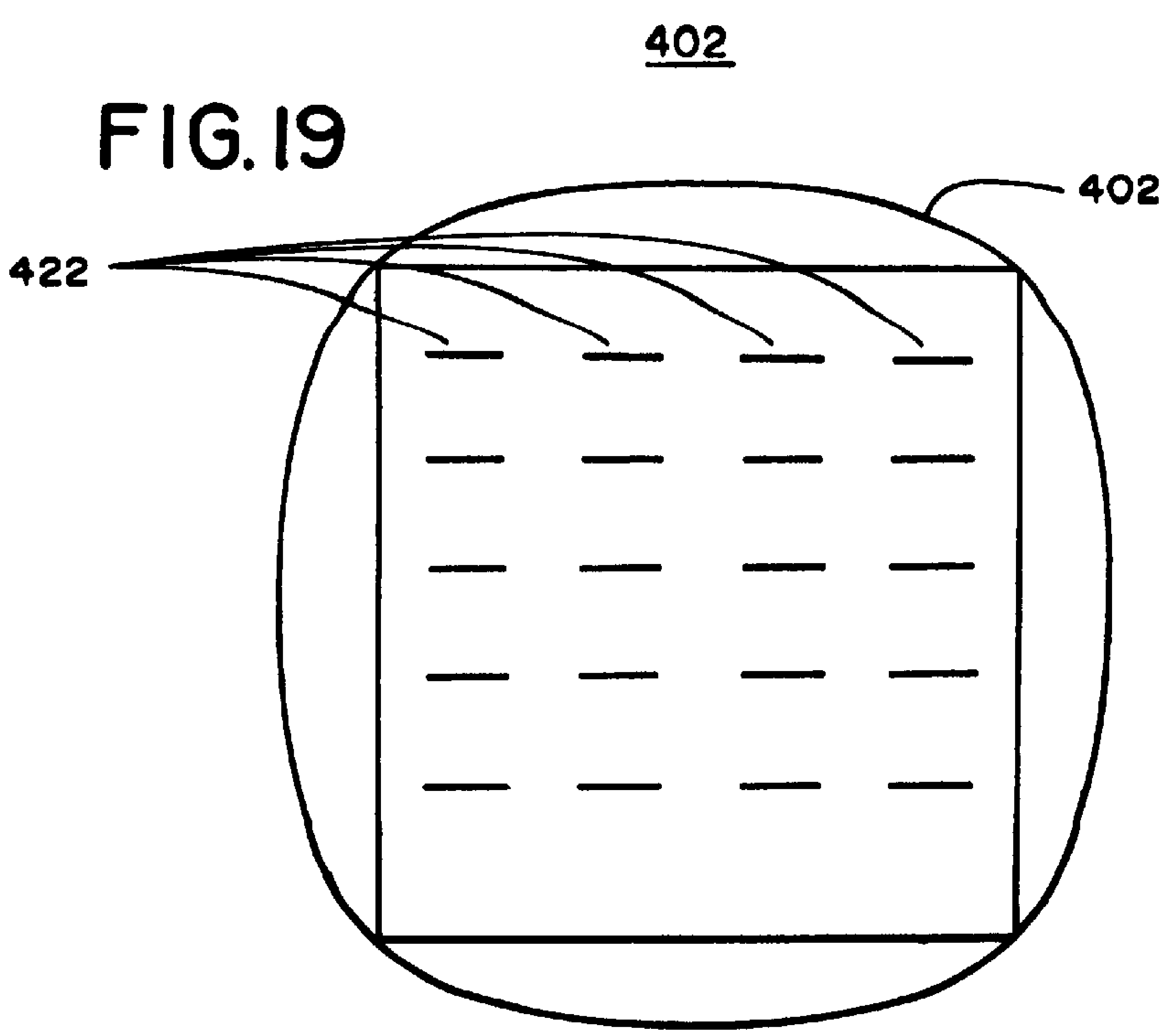

400
VERTICAL SCAN CIRCUIT
406
402
410
412
AMPLITUDE MODULATION
408
404
HORIZONTAL SCAN CIRCUIT

FIG. 19


402
402
422

DNA SEQUENCING AND DNA TERMINATORS

REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. patent application Ser. No. 08/275,232, filed Jul. 14, 1994, abandoned, which is a divisional application of U.S. application Ser. No. 07/950,734, filed Sep. 24, 1989, now U.S. Pat. No. 5,346,603, which is a file wrapper continuation of Ser. No. 07/799,712 filed Nov. 6, 1991 (abandoned) which is a file wrapper continuation of Ser. No. 07/632,605, filed Dec 24, 1990, (abandoned), which is a file wrapper continuation of Ser. No. 07/078,279, filed Jul. 27, 1987 (abandoned), which is a divisional of U.S. application Ser. No. 06/594,676, filed Mar. 29, 1984, in the names of Lyle Richard Middendorf and John Brumbaugh now U.S. Pat. No. 4,729,947, and assigned to the same assignee as this application and is a continuation-in-part of U.S. patent application Ser. No. 08/288,461 filed Aug. 10, 1994 (now U.S. Pat. No. 5,534,125), which is a division of U.S. patent application Ser. No. 08/018,806, filed Feb. 17, 1993 now U.S. Pat. No. 5,360,523, which is a division of U.S. application Ser. No. 07/763,230, filed Sep. 20, 1991, (now U.S. Pat. No. 5,230,781) and of U.S. application Ser. No. 07/570,503 filed Aug. 21, 1990, (now U.S. Pat. No. 5,207,880) which are continuations in part of Ser. No. 07/078,279 filed Jul. 27, 1987, which is a division of U.S. application Ser. No. 06/594,676 for DNA SEQUENCING filed by Middendorf et al., on Mar. 29, 1984, and assigned to the same assignee as this application, now U.S. Pat. No. 4,729,947, and a continuation-in-part of U.S. application Ser. No.08/204,627, filed Mar. 1, 1994, now U.S. Pat. No. 5,571,388 which is a continuation-in-part of U.S. application Ser. No. 07/860,140, filed Mar. 30, 1992, now U.S. Pat. No. 5,366,603 which is a division of U.S. application Ser. No. 07/763,230 filed Sep. 20, 1991, now U.S. Pat. No. 5,230,781, which is a continuation-in-part of U.S. application Ser. No. 07/570,503 filed Aug. 21, 1990, now U.S. Pat. No. 5,207,880, which is a continuation-in-part application of Ser. No. 07/078,279 filed Jul. 27, 1987, which is a division of U.S. application Ser. No. 06/594,676 for DNA SEQUENCING filed by Middendorf et al., on Mar. 29, 1984, and assigned to the same assignee as this application, now U.S. Pat. No. 4,729,947.

BACKGROUND OF THE INVENTION

This invention relates to DNA terminators for signaling the end of DNA strand synthesis which may be used to prepare DNA strands and for sequencing and to the marking and sequencing of DNA strands.

In one class of techniques for sequencing DNA, identical strands of DNA are marked. The strands are separated into four aliquots. The strands in a given aliquot are either individually cleaved at or synthesized to any base belonging to only one of the four base types, which are adenine, guanine, cytosine and thymine (hereinafter A, G, C and T). The adenine-, guanine-, cytosine- and thymine-terminated strands are then electrophoresed for separation. The different marked strands are detected and the terminating base identified for each. The rate of electrophoresis of the different terminated base strands indicates the DNA sequence.

In one class of technique for marking DNA strands, the strands are marked with a fluorescent dye. The strands are marked by: (1) hybridzing specially synthesized fluorescently marked oligonucleotide primer strands to template strands of DNA and then extending the primer strands with DNA polymerase to incorporate unmarked deoxy nucleotide triphosphates and an unmarked terminator such as a dideoxynucleotide triphosphate for the purpose of uniquely terminating strand synthesis; (2) hybridizing unmarked oligonucleotide primer strands to template strands and then extending the primer strands with DNA polymerase to incorporate marked deoxynucleotide triphosphates and an unmarked terminator such as a dideoxy nucleotide triphosphate for the purpose of uniquely terminating strand syntesis; or (3) hybridizing unmarked oligonucleotide primer strands with DNA polymerase to incorporate unmarked deoxynucleotide triphosphates and a marked terminator such as a dideoxy nucleotide triphosphate for the purpose of uniquely terminating strand synthesis.

In a prior art technique of this class, the marked strands are of four types, each corresponding to the appropriate one of adenine-, guanine-, cytosine-, and thymine-terminated strands. Moreover, in prior art sequencing techniques of this class, the fluorescent dyes used as markers have their maximum emission spectra in the visible range with wavelengths shorter than 650 nm, the DNA is subject to irradiation in this visible spectra, and visible spectra detectors and light sources are used. Generally photomultipliers tubes are used for detection and gas lasers such as Argon-ion or Helium—Neon lasers are used as light sources.

Cyanine dyes are known to absorb far red (600–700 nm) and near infrared (700–1200 nm) light and techniques for the synthesis of derivatives of the cyanine dyes are known. The use of cyanine dyes as markers for DNA sequencing has been successfully achieved as described by Middendorf, L. R., et al., "Electrophoresis Journal" 13, 487–494 (1992).

An additional disadvantage of the prior art techniques for DNA sequencing is the need to have four different terminators, regardless of whether or not they are marked, for the purpose of providing a unique termination of strand synthesis.

In a prior art sequencing technique, the DNA strands are marked with a radioactive marker, and after being separated by electrophoresis, film is exposed to the gel and developed to indicate the sequence of the bands. The range of lengths and resolution of this type of static detection is limited by the size of the apparatus.

The prior art techniques for DNA sequencing have several disadvantages such as: (1) because the dyes have their emission spectra in that visible region of the light spectrum having a wavelength shorter than 650 nm, the lasers used to excite the fluorescent markers, and under some circumstances, the detectors for the light tend to be expensive; (2) the signal information is relatively noisy due to the background fluorescence interference by biomolecules and glass; (3) they are relatively slow; (4) they are at least partly manual; (5) they are limited to relatively short strands of DNA; (6) they do not have high sensitivity; and (7) they required complex protocols involving multiple types of synthesis terminators.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the invention to provide a novel technique for DNA termination.

It is a further object of the invention to provide a novel technique for marking DNA strands.

It is a still further object of the invention to provide a novel technique for DNA sequencing.

It is a still further object of the invention to provide a novel technique for terminating DNA strand synthesis.

It is a still further object of the invention to provide a novel universal terminator for DNA sequencing.

It is a still further object of the invention to provide novel apparatuses and methods for sequencing relatively large fragments of DNA.

It is a still further object of the invention to provide novel apparatuses and methods for sequencing DNA fragments of 100 bases or more.

It is a still further object of the invention to provide a technique for continuous sequencing of DNA.

It is a still further object of the invention to continuously sequence DNA without the spatial limitations of range of lengths and resolution.

It is a still further object of the invention to provide a novel technique for continuously sequencing DNA using fluorescent detection.

It is a still further object of the invention to provide a novel technique for continuously sequencing DNA marked with fluorescence which more clearly distinguishes marked DNA fragments from background fluorescence.

It is a still further object of the invention to provide a novel technique for scanning fluorescent material.

It is a further object of the invention to provide novel equipment and methods for the sequencing of far red and/or infrared but especially far red (600–700 nm) and/or near infrared (700–3000 nm) fluorescence labeled DNA and the detection of the DNA after irradiation by light from laser diodes.

It is a still further object of the invention to provide a novel technique for DNA sequencing using a novel fluorescent marker attached to the DNA.

It is a still further object of the invention to provide a novel fluorescent marker, method of synthesizing the marker and of attaching it to DNA.

It is a further object of the invention to provide a novel method, marker and equipment that permits the detection of marked molecules, including DNA, using diode light sources such as laser diodes or light emitting diodes and semiconductor detectors such as photodiodes or charge-coupled devices (CCD's).

In accordance with the above and further objects of the invention, strands of DNA are prepared and continuously electrophoresed and identified. For this purpose, the strands are fluorescently marked. The light emitted while irradiating the strands near the terminal end of the electrophoresis channel is detected and correlated. The electrophoresis conditions are selected so that strands being electrophoresed near the terminal end of the electrophoresis channel are fully resolved prior to the resolution of longer strands that have not yet reached the terminal end of the electrophoresis channel, and so on, in a continuous process over a period of time.

The gel size, electric field and DNA mobilities are such that the first bands to be moved completely through the gel are fully resolved while the last bands are yet unresolved in a continuous process controlled to cause at least ten percent of the bands to be resolved and electrophoresed through the gel while the less mobile bands are yet unresolved near the entrance end of the gel. These less mobile bands become resolved little by little over time in a continuous fashion without interruption of the movement of these bands through the gel.

In one embodiment for labeling strands, a substrate that terminates synthesis is labeled with a marker. In another embodiment, the strand is labeled either by a labeled primer that provides the initial portion of the strand or by a labeled deoxy nucleotide triphosphate that allows labeling of the strand during elongation. In any of these embodiments, it is preferable for the substrate that terminates synthesis to have a heterocycle other than naturally occuring DNA heterocycles such as adenine, cytosine, guanine and thymine. Alternatively, the substrate may have a modified base or sugar or be missing either the base or sugar (or both structures).

In terminating the strand synthesis, a universal terminator substrate (which may or may not be marked) is used. In this specification, the word "universal" as applied to terminator substrates means a terminator substrate that can be incorporated by DNA polymerase at any A, G, T or C position. In this specification the word "terminator" means a substrate that once incorporated by the DNA polymerase into a growing DNA strand, it will cause termination of the DNA polymerase activity on that strand.

One step in terminating strand synthesis is to design a universal terminator that has a lower incorporation efficiency than a normal "A", "C", "G", "T" deoxynucleotide. In this specification "a lower incorporation efficiency" means that the DNA polymerase reactivity for normal deoxynucleotides is at least five times higher than for the universal terminator substrate. Incorporation efficiency of a substrate is related to the relative reactivity of a DNA polymerase with respect to normal deoxynucleotide triphosphates as compared to the reactivity of a polymerase to the substrate. For example the reactivity of Taq polymerase is about 10,000 times higher for deoxynucleotide triphosphates than for dideoxy nucleotide triphosphates. For Sequenase polymerase (Sequenase is a trademark of U.S. Biochemical Corporation) the reactivity is between one and five times higher for deoxynucleotide triphosphates than for dideoxy nucleotide triphosphates.

In terminating strand synthesis, the hybridized primer-template is split into four aliquots. In the "A" vial is added DNA polymerase and normal amounts of "C", "G", and "T" deoxynucleotides, along with a reduced amount of "A" deoxynucleotide and an amount of the universal terminator such that statistically the universal terminator has less than a one percent chance of being incorporated at sites where a "C", "G", or "T" deoxynucleotide should be incorporated and about a one percent chance of being incorporated at sites where an "A" deoxynucleotide should be incorporated. The amount of "A" deoxynucleotide and universal terminator added may be modified to give a different chance than one percent depending on the desired length of the terminated strand as well as accomodate universal terminator substrates that have equivalent incorporation efficiencies as normal deoxynucleotides. No "A", "C", "G" or "T" dideoxynucleotides are used. In one embodiment the primer is marked. In another embodiment one or more of the deoxynucleotides is marked. In still another embodiment the terminator is marked.

At each position of adding substrate, if a "C", "G", or "T" deoxynucleotide is to be added, it is preferentially added rather than the universal terminator (even though the universal terminator theoretically could be added at any "A", "C", "G" or "T" position) due to the much lower incorporation efficiency by the DNA polymerase for the universal terminator and/or a reduction in the amount of universal terminator present in the sequencing reaction. However, at each position where an "A" is to be added, the DNA polymerase may statistically incorporate either the "A" deoxynucleotide or the universal terminator since the "A" deoxynucleotide concentration is reduced. The result is a batch of differing length strands, each of which is terminated by the universal terminator at any one of the "A" positions.

Similar strategies are followed for the "C", "G", and "T" vials, except that the amount of "C", "G" and "T" deoxynucleotides are reduced for their respective vial. The same type of universal terminator is used in all four vials. In the preferred embodiment, the marked strands are electrophoresed in a continuous, on-line process and detected.

In one embodiment, the primers are far red or near infrared wavelength fluorescent labeled DNA and the detection of the DNA after irradiation by far red or near infrared light from a laser diode is accomplished using a novel label attached to the primer. In this embodiment only one type of labeled primer and only one type of universal terminator is required for the "A", "C", "G" and "T" reactions. An extention of this embodiment employs multiple types of labels differentiated by optical characteristics such as absorbance wavelength, emission wavelength, fluorescence lifetime, or a combination of such. Thus, multiple types of primer labeled strands can be optically differentiated even though all strands are terminated with the identical type of universal terminator.

In a second embodiment the primers are unlabeled and the universal terminator is marked, thus it is a universal labeled terminator. Again, only one type of unlabeled primer and only one type of universal labeled terminator is required for the "A", "C", "G" and "T" reactions. An extension of this embodiment employs multiple types of labels differentiated by characteristics such as absorbance wavelength, emission wavelength, fluorescence lifetime, or a combination of such. Thus, each set of "A", "C", "G" and "T" strands uses one unique universal labeled terminator. Sets are discriminated by the specific optical uniqueness of the universal labeled terminator used to create each set.

The strands of DNA are continuously electrophoresed and identified for DNA sequencing. To aid in identification, the strands are marked with fluorescent labels that emit light in the far red (600–700 nm) or near infrared (700–3000 nm) region. The strands are irradiated with light in the far red or near infrared region and the light emitted from the fluorescent labels is detected and used to obtain information about the DNA strands. In one embodiment, the strands are detected by irradiating with a far red or near infrared laser diode light source.

The detection apparatus includes a laser diode that emits light of a wavelength near or in the optimum absorbance spectrum of the marker. In the preferred embodiment, the light source is a laser diode that irradiates channels of DNA strands with far red or near infrared light having a wavelength that matches the absorbance region of the marker. The detector includes a light sensor which is preferably an avalanche photodiode sensitive to the far red or near infrared light emission of the marker. It may include a filtering system having a pass band suitable for passing selectively the optimum emission of the fluorescent marker to the light sensor.

The photodiode, photomultiplier or other light sensor selectively detects the fluorescence, using techniques which enhance the signal/noise ratio. One technique is to modulate the laser source by pulsing the electrical current driving the laser, with detection facilitated by a lock-in amplifier. The detection is made in a wavelength band including the high emission spectrum of the fluorescent marker.

The apparatus for such continuously sequencing of DNA includes one or more electrophoresis channels, each adapted to receive fluorescently labeled DNA strands, having at one end a universal terminator substituted for a base of a given type.

To provide marking, either a fluorescent marker is attached to the DNA fragments prior to their being electrophoresed, or probes are used to combine or hybridize with the DNA strands. In the latter case, the detection is accomplished by detecting a fluorescent marker that is chemically attached to the probe. In the preferred embodiment, the marker is a dye that fluoresces in the far red or near infrared region.

The electrophoresis may be provided in conventional gel slabs or in tube gels such as gel filled capillary tubes or buffer filled capillary tubes which avoid the need for gel and make the cleaning more convenient.

For the configuration using conventional gel slabs, one embodiment provides for a different input section for each of four channels that are for a corresponding one of the A, G, T and C strands. Other embodiments allow for less than four gel channels by judiciously combining one or more base types A, G, T, or C in a channel. The strands are detected during electrophoresis either in the gel by scanning back and forth across the gel at a fixed distance from the entrance end of the gel or by one or more fixed detectors located at a fixed distance from the entrance end of the gel or after leaving the gel. The strands are detected in a manner that indicates their mobility in the gel to indicate the sequence of the A, G, C and T strands of different lengths.

The detection apparatus includes a light source, such as a laser diode or light-emitting diode or other suitable source that emits light in the optimum absorbance spectrum of the marker. The light may be split by the use of fiber optics or other conventional optical components so there is a light source for each channel.

In the preferred embodiment, the light source is a laser diode that irradiates the channels with far red or near infrared light having a wavelength that matches the absorbance region of the marker. The detector includes a light sensor which is preferably an avalanche photodiode sensitive to the far red or near infrared light emission of the marker. It may include a filtering system having a pass band suitable for passing selectively the optimum emission of the fluorescent marker to the light sensor.

Correlation of the channel in which the fluorescent light is detected and the time of detection of the fluorescent light indicates: (1) if the type of universal termination is A, G, C or T or a combination thereof for those embodiments which have more than one base type in a channel; and (2) the time sequence of separation of each strand in each channel of the electrophoresis gel. This information, in turn, indicates the overall sequence of strands.

To use the apparatus to sequence DNA strands, identical DNA strands are normally formed of a length greater than 100 bases. In one embodiment, the strands are marked by a suitable marker at one end. The strands are divided into four aliquots and the strands within each aliquot are synthesized to any base belonging to a specific base type. These four aliquots are then electrophoresed through one or more identical channels to separate strands so that the shorter strands are resolved towards the end of the gel prior to resolution of the longer strands, which may be near the entrance end of the gel at the time the shorter strands are being resolved. This occurs in a continuous process so a substantial number of different length strands may be resolved in a relatively short gel. This methodology takes advantage of time-resolved bands, as opposed to the limitations of spatial-resolved bands.

The gel size, electric field and DNA mobilities are such that the more mobile bands are fully resolved while the less mobile bands are yet unresolved in a continuous process such that at least ten percent of the bands have been resolved by electrophoresis in the gel while the less mobile bands which are near the entrance end of the gel are not fully resolved. These less mobile bands become resolved little by little over time in a continuous fashion without interruption of the movement of these bands through the gel. The markers are detected by transmitting far red or near infrared light to fluorescently marked DNA strands.

To obtain maximum information in those embodiments in which a gel slab is scanned, a microscope and laser are moved together on a platform with respect to the gel. To ensure parallelism between the microscope/laser assembly and the slab gel as well as optimal focusing of the assembly onto the slab gel, in one embodiment, the sensor determines the point in the glass-gel-glass sandwich having minimal fluorescence and focuses on it. This minimal fluorescence is due to the reduced fluorescence of the gel as compared to the glass. The microscope is continually moved and refocused as scanning takes place to maintain the optimum focus.

In another scanning embodiment, the microscope determines the optimal focus position at one location on the glass-gel-glass sandwich and then is moved to another location on the glass-gel-glass sandwich where an optimal focus position is determined for that location. The scanning mechanism is then pivoted so that the scanning mechanism is parallel to a line connecting the two optimal focus positions previously determined. This insures that the gel slab is in the focal plane of the microscope/laser assembly. The intensity signal received from the scanning microscope/laser assembly which indicates the presence or absence of DNA strands is directly transmitted to the intensity input of the computer monitor so that the display varies in brightness rather than providing an amplitude trace.

For purposes of focusing, either by pivoting the scanning mechanism or by adjusting the microscope at different points during a scan to follow an established line, the microscope/laser assembly and the plane of the glass-gel-glass sandwich move orthogonally with respect to each other at one end of the scan in order to locate the position of lowest fluorescence, which is a location in the gel slab between the two glass plates. Then the microscope/laser assembly moves to the other end of its scan and performs the same function. These two focusing movements are utilized to move the scan mechanism in one embodiment and to program the movement of the microscope focus in another embodiment so that the microscope in the one embodiment moves continuously along a single line and the scan mechanism has been prepositioned such that that line is parallel to the gel slab, and in the other embodiment, the focus is changed at six points to accommodate a non-parallel alignment between the scan mechanism and the gel.

From the above summary, it can be understood that the sequencing techniques of this invention have several advantages, such as: (1) they take advantage of resolution over time, as opposed to space; (2) they are continuous; (3) they are automatic; (4) they are capable of sequencing or identifying markers in relatively long strands including strands of more than 100 bases; (5) they are relatively economical and easy to use; (6) they permit efficient focusing of a light sensor onto the DNA bands; (7) they provide an easy to observe display; (8) because the dyes have their emission spectra in the far red or near infrared light spectrum, small inexpensive far red or near infrared laser diodes may be used; (9) the signal information is characterized by relatively low noise; and (10) they provide a simple protocol using universal termination of strands elongated by DNA polymerase.

SUMMARY OF THE DRAWINGS

The above noted and other features of the invention will be better understood from the following detailed description when considered with reference to the accompanying drawings in which:

FIG. **1** is a block diagram of an embodiment of the invention;

FIG. **2** is one embodiment of a portion of the DNA sequencing apparatus of FIG. **1**;

FIG. **3** is another embodiment of a portion of the DNA sequencing apparatus of FIG. **1**;

FIG. **4** is a block diagram of an embodiment of the invention;

FIG. **5** is a perspective view of a portion of the embodiment of FIG. **4**;

FIG. **6** is a sectional view taken through lines **6—6** of FIG. **5**;

FIG. **7** is a sectional view of a portion of FIG. **5** taken through lines **7—7**;

FIG. **8** is an exploded perspective view of a portion of the embodiment of FIG. **7**;

FIG. **9** is an enlarged view, partly broken away, of a portion of the embodiment of FIG. **7**;

FIG. **10** is a block diagram of a circuit that may be used for coordination of a sensor, scanner drive and laser used in the embodiment of FIG. **7**;

FIG. **11** is a perspective view from the top right side of another embodiment of scanning section usable in the DNA sequencing apparatus of FIGS. **4** and **5**;

FIG. **12** is a perspective view from the bottom right side of the sequencing system of FIG. **11**;

FIG. **13** is a flow diagram of a program used to control the operation of the system of FIG. **11** and FIG. **12**;

FIG. **14** is a more detailed flow diagram of the software control for a portion of the program of FIG. **13**;

FIG. **15** is a schematic diagram of another portion of the program of FIG. **13**;

FIG. **16** is a block diagram of another embodiment of the program of FIG. **15**;

FIG. **17** is a block diagram of a control portion for the embodiments of FIGS. **1–16**;

FIG. **18** is a schematic diagram of a display arrangement useful in the embodiment of FIGS. **1–17**; and

FIG. **19** is a front view of a display screen resulting from its use of the display arrangement of FIG. **18**.

DETAILED DESCRIPTION

In FIG. **1**, there is shown a simplified block diagram of a DNA sequencing system **10**. In this embodiment, the DNA sequencing system **10** includes containers for treatment of the DNA in accordance with the method of Sanger described by F. Sanger, S. Nicklen and A. R. Coulson, "DNA Sequencing with Chain-Terminating Inhibiters," *Proceeding of the National Academy of Science, USA,* Vol. 74, No. 12, 5463–5467, 1977 indicated in the embodiment 10 of FIG. **1** at **20**A, **20**G, **20**C and **20**T shown as a group generally at **12**.

In this method, DNA strands are used as templates to synthesize DNA with synthesis terminating at given base types A, G, C or T in a random manner to obtain a plurality of different molecular weight strands. The limited synthesis is obtained by using nucleotides which will terminate synthesis and is performed in separate containers, one of which has the special A nucleotide, another the special G nucleotide, another the special C nucleotide and another the special T nucleotide. These special nucleotides may be dideoxy nucleotides or marked nucleotides, both of which would terminate synthesis. So, each of the four batches will be terminated at a different one of the types of bases A, G, C and T randomly.

In one embodiment for labeling strands, a substrate that terminates synthesis is labeled with a marker. In another embodiment, the substrate that terminates synthesis is unlabeled but the strand is labeled either by a labeled primer that provides the initial part of the strand or by a labeled deoxy nucleotide triphosphate that allows labeling of the strand during elongation. In either embodiment, it is preferable for the substrate that terminates synthese to have a heterocycle other than naturally occuring DNA heterocycles such as adenine, cytosine, guanine or thymine. Additionally, the substrate may have a modified base or sugar (or both structures).

In the preferred embodiment for terminating the strand synthesis, a universal terminator substrate (which may or may not be marked) is designed to have a lower incorporation efficiency than a normal "A", "C", "G", "T" deoxynucleotide. In this specification "a lower incorporation efficiency" as applied to a universal terminator substrate means that the DNA polymerase reactivity for normal deoxynucleotides is at least five times higher than for the universal terminator substrate. In another embodiment, the incorporation efficiency of the universal terminator is nearly the same as for normal deoxynucleotides. In this embodiment, the amount of universal terminator used in a Sanger sequencing reaction is reduced.

Incorporation efficiency of a substrate is related to the relative reactivity of a DNA polymerase with respect to normal deoxynucleotide triphosphates as compared to the reactivity of a polymerase to the substrate. For example, the reactivity of Taq polymerase is about 10,000 times higher for deoxynucleotide triphosphates than for diodeoxynucleotide triphophates. For Sequenase polymerase (sequenase is a trademark of U.S. Biochemical Corporation) the reactivity is between one and five times higher for deoxynucleotide triphosphates than for dideoxynucleotide triphosphates. In this specification, the word "universal" as applied to terminator substrates means a terminator substrate that can be incorporated by DNA polymerase at any A, G, T or C position. In this specification, the word "terminator" means a substrate that once incorporated by the DNA polymerase into a growing DNA strand, it will cause termination of the DNA polymerase activity on that strand.

In terminating strand synthesis, the hybridized primer-template is split into four aliquots. In the "A" vial is added DNA polymerase and normal amounts of "C", "G", and "T" deoxynucleotides, along with a reduced amount of "A" deoxynucleotide and an amount of the universal terminator such that statiscally the universal terminator has less than a one percent chance of being incorporated at sites where a "C", "G", or "T" deoxynucleotide should be incorporated, and about a one percent chance of being incorporated at sites where an "A" deoxynucleotide should be incorporated.

The amount of "A" deoxynucleotide and universal terminator added may be modified to give a different chance than one percent depending on the desired length of the terminated strand as well as accomodate universal terminator substrates that have equivalent incorporation efficiencies as normal deoxynucleotides. No "C", "G" or "T" dideoxynucleotides are used. In the first embodiment, the universal terminator is marked. In the second embodiment, the universal terminator is unmarked, and the strand is marked either prior to elongation by DNA polymerase of a labeled primer oligonucleotide, or during elongation by the DNA polymerase through the incorporation of a marked "C", "G" or "T" deoxynucleotide.

At each position of adding substrate, if a "C", "G", or "T" deoxynucleotide is to be added, it is preferentially added rather than the universal terminator (even though the universal terminator theroretically could be added at any "A", "C", "G" or "T" position) due to the lower incorporation efficiency by the DNA polymerase for the universal terminator and/or a reduced amount of universal terminator present in the sequencing reaction. However, at each position where an "A" is to be added, the DNA polymerase may statistically incorporate either the "A" deoxynucleotide or the universal terminator since the "A" deoxynucleotide concentration is reduced. The result is a batch of differing length strands, each of which is terminated by the universal terminator at any one of the "A" positions.

Similar strategies are followed for the "C", "G", and "T" vials, except that the amount of "C", "G" and "T" deoxynucleotides are reduced for their respective vial. The same type of universal terminator is used in all four vials. The strands are loaded into four electrophoresis channels and continuous, on-line detection is used.

In the second embodiment, the Sanger sequencing protocol for a given DNA template uses labeled primers and also requires only one type of universal terminator dd*TP for all the four reactions: (where * indicates a universal base—usually a heterocyclic compound as a substitute for the adenine, cytosine, guanine or thymine base). (In the first embodiment the dd*TP is marked with dye label and the primer is not labeled). In these four reactions of the second embodiment: (1) the "A" reaction has the following components: a template, a labeled primer, a buffer, dCTP, dGTP, dTTP, an adjusted amount of dATP and dd*TP and a DNA polymerase enzyme; (2) the "C" reaction has the following components: a template, a labeled primer, a buffer, dATP, dGTP, dTTP, an adjusted amount of dCTP and dd*TP and a DNA polymerase enzyme; (3) the "G" reaction has the following components: a template, a labeled primer, a buffer, dATP, dCTP, dTTP, an adjusted proportional amount of dGTP and dd*TP and a DNA polymerase enzyme; and (4) the "T" reaction has the following components: a template, a labeled primer, a buffer, dATP, dCTP, dGTP, an adjusted amount of dTTP and dd*TP and a DNA polymerase enzyme.

Heterocycles other than naturally occuring DNA heterocycles such as adenine, cytosine, guanine and thymine are incorporated into the universal terminator dd*TP. These alternate heterocycles have reduced hydrogen bonding and have preferences to hydrogen bond with all four bases. One suitable dd*TP is dideoxy inosine triphosphate, a purine. Dideoxy inosine triphosphate can generally incorporate in the place of all four bases. Suitable universal terminators are shown in formula 2 for dideoxy inosine triphosphate; formula 1 for 5-nitro indole analog and formula 3 for 3-nitro pyrrole analog, where TPO is the triphosphate.

In FIG. **2**, there is shown another embodiment B26A of gel electrophoresis having a negative-potential buffer for the A channel indicated generally at B29A, a gel electrophoresis channel for A terminated DNA indicated at B27A and a positive potential buffer for the channel indicated at B31A. This embodiment is intended to provide a small diameter cylindrical gel for each channel so as to permit better temperature control and thus control migration rate and reduce diffusion caused by temperature gradients such as may occur across a thicker slab gel.

For this purpose, the channel B27A includes a 0.01 to a 1 millimeter inner diameter glass column such as a chromatographic columm indicated at **33** with a gel inside of it. The gel may be cross-linked acrylamide or a non-crosslinked matrix. The column **33** is of sufficient length to separate the DNA.

At one end of the channel B27A is the buffer B29A adapted to provide a buffer solution in a surrounding cup shaped container **45**, which buffer extends over the entering end **37** and contains within it a negative voltage electrode **47**. At the exit end, there is similarly mounted a buffer compartment **51** containing buffer which is grounded by an electrode **53** and immerses in buffer the exit end **35** of the gel column B27A. It may be shaped with a reducing orifice ending in a micro-orifice **55** at its lower end to permit the flow of buffer therethrough containing DNA which emerges from exit end **35** for detection. To supply new buffer, a buffer reservoir **57** is connected through a pump **59** to the top of the buffer **51**.

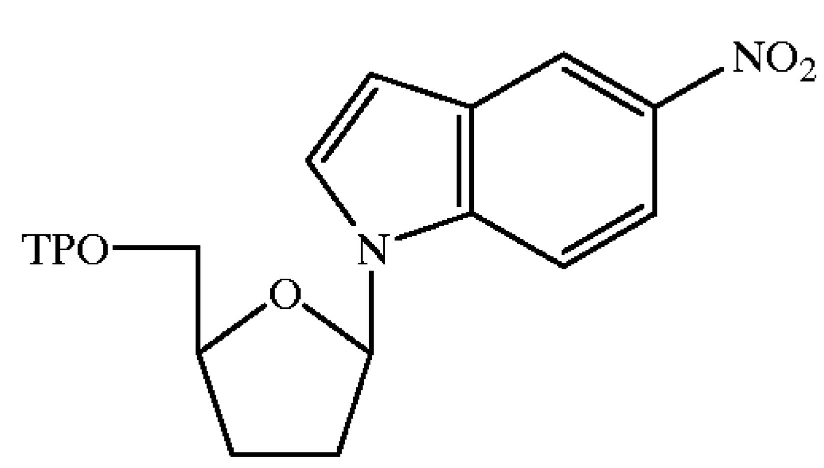
In FIG. **3**, there is shown still another embodiment C26A which includes capillary columns **61** and **63** as commonly used in capillary electrophoresis. These columns may be filled with buffer solution rather than a gel and be used for electrophoresis. The bands of A, G, C or T type bases might flow through a different one of multiple parallel capillaries, or they might flow together through only one capillary.

The separation path such as gel channels or capillary tube length should be no longer than two meters for range of lengths of DNA for 50 to 10,000 or more bases. However, as the range of DNA lengths increase, the time required increases. Also, the time required for each separation is in the range of from ½ second to 5 minutes for each added base of length separation.

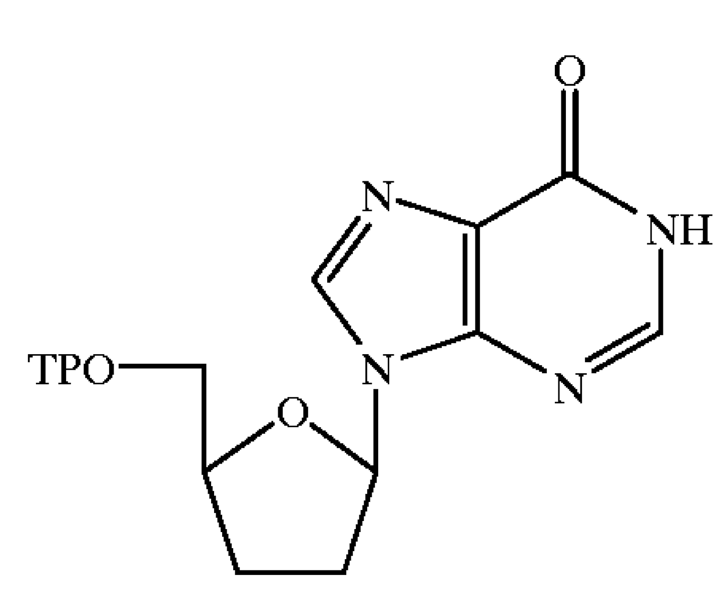
In the operation of sequencing DNA, DNA strands with bases above 100 in number are marked with one or more fluorescent molecules and separated in accordance to the size of the fragment. The bands are then detected by light.

To separate the fragments, the marked DNA fragments are electrophoresed through gel or liquid in one or more channels or columns. The DNA fragments separate in accordance with their length during electrophoresis. Thus the fastest migrating fraction is the fragment which is synthesized to the first base closest to the primed end of the strand and, since in the preferred embodiment, the channels are separate, it is known from the channel which

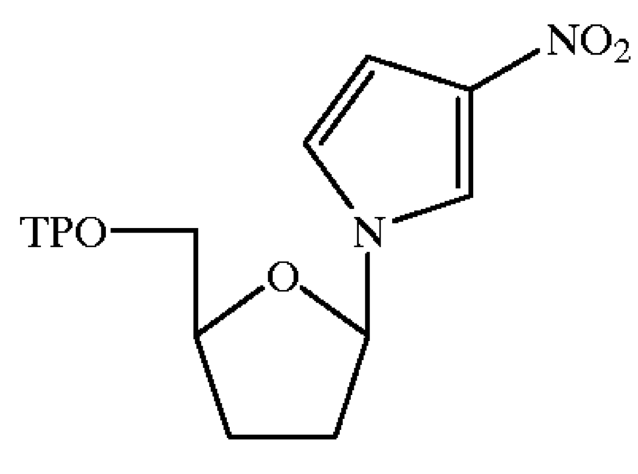
FORMULA 1



FORMULA 2



FORMULA 3



base A, G, C or T is the first one in the sequence. The next band in time in the gel is the fragment which is one base longer than the first one since it encompasses both the first base and the second one from the primed end of the DNA strand. Similarly, the third fragment to form a band during electrophoresis encompasses the first three base units and so on.

Because a large number of bases are used, there is a large number of varying length fragments and the amount of fragments in each band is relatively low. Thus, the gel and the field must be selected to provide a band having low diffusion, high concentration density and adequate separation for detection. The gel slab or capillary is sufficently long such that the first bands to be moved completely through the gel are fully resolved while the last bands are unresolved in a continuous process. More specifically, at least 10 percent of the bands are resolved and electrophoresed through the gel while the least mobile bands are yet unresolved near the entrance end of the gel.

The sequencing of far red or near infrared fluorescence labeled DNA and the detection of the DNA after irradiation by far red or near infrared light from a laser diode is accomplished using a far red or near infrared label prepared for this purpose and either directly attached to the DNA primer oligonucleotides or deoxynucleotides or the universal terminator. In this specification the word "infrared" will be used at times to include far red wavelengths (600–700 nm) and near infrared (700–3000 nm). The strands of DNA are continuously electrophoresed and identified for DNA sequencing.

The strands are marked with fluorescent labels that have their maximum fluorescense and their maximum absorbance at wavelengths of light in the far red or near infrared region. The strands are irradiated with light in the far red or near infrared region from a laser diode and the light emitted from the fluorescent labels is detected and used to obtain information about the DNA strands. The detector includes a light sensor which is preferably an avalanche photodiode sensitive to the far red or near infrared light emission of the marker. It may include a filtering system having a pass band suitable for passing selectively the optimum emission of the fluorescent marker to the light sensor.

To mark the DNA strand, a dye is synthesized having the desired properties or a known dye is modified. In the preferred embodiment a novel dye having the preferred absorbance spectrum, high molar absorptivity and fluorescence properties, and at least one reactive group enabling coupling to DNA primers, deoxynucleotides or terminators is synthesized.

The dye is synthesized or modified from a known dye or selected from commercially available sources to have an absorbance band and an emission band within the desired region. In the preferred embodiment this region is a region encompassing the far red or near infrared region when attached to a primer oligonucleotide, deoxynucleotide or universal terminator. The dye should provide high quantum yield in an optical band selected to reduce background fluorescence noise. The preferred dyes for many applications calling for the labeling of biomolecules are cyanine dyes having an NCS (isothiocyanate) group on the dye that may react with the primary amine group of the biomolecule to form a thiourea linkage or having NHS esters or hydroxyl groups or carboxyl groups for the purpose of reacting with the biomolecule to form linkages.

In the preferred embodiment, cyanine dyes are synthesized. The preferred dyes are pentamethine or heptamethine cyanines which efficiently absorb light having wavelengths in the region of 630 to 900 nm (nanometers) (maximum absorbance wavelength). This wavelength is suitable for reducing background fluorescence in DNA sequencing and corresponds to the radiation of diode lasers made of such materials as GaAlAs, GaAs, InGaAlP, GaInP, AlGaAs, AlGaInP, GaAlP, InGaAsP, GaInP/AlInP, or GaInP/AlGaInP. The GaAlAs diode, for example, emits at 780–800 nm and is used for irradiating the gel electrophoresis slab sandwich, column, or capillary used for DNA sequencing. Formulas 4–6 are typical synthesized dyes and formula 7 is a suitable modified dye.

Formula 4 shows synthesized cyanine dyes having NCS (isothiocyanate) as a reactive group for attachment of a biomolecule. In this embodiment, when X is H, the maximum absorbance wavelength is 787 nm in methanol and 801 nm in DMSO, and the maximum emission wavelength is 807 nm in methanol and 818 nm in DMSO. When X is —OCH_3, the maximum absorbance wavelength is 786 nm in methanol and the maximum emission wavelength is 806 nm methanol. In both cases, the quantum yield is greater than 15 percent.

Formula 5 shows synthesized cyanine dyes which have a high quantum yield in methanol around 35 percent. When R is ethyl or sulfonatobutyl and n is either 1 or 2, the maximum absorbance wavelength is between 762 and 778 nm. Depending on the solvent, the maximum emission wavelength is between 782 and 800 nm.

In the synthesized dye represented by formula 6, the maximum absorbance wavelength is between 773 and 778 nm depending on the solvent and the maximum emission wavelength is between 789 and 806 nm depending on the solvent. The quantum yield is between 25 percent and 35 percent depending on the solvent.

A new series of dyes is obtained by modifying a commercial dye, IR-144 shown as formula 7, with R being

—CH_2—CH_3. The series of dyes, obtained by changing R, is close to having the desired wavelength of maximum fluorescence. The wavelength of maximum absorbance may be modified by changing the functional group R. The unmodified dye may be obtained from Laboratory and Research Products Division, Eastman Kodak Company, Rochester, N.Y., 14650. It is advertised in Kodak publication JJ-169.

In the preferred embodiment, the fluorescence maximum wavelength is about 819 nanometers and the detector is adjusted to receive this wavelength and not others by appropriate filtering. The absorbance maximum is selected to be different and to correspond to the preferred available laser diode emission. For example, in this formula, R may be any of the following four groups, depending on the desired wavelength of the absorbed light, which are:

(1) —CH_2—CH_2—OH for an absorbance wavelength of 796 nanometers;

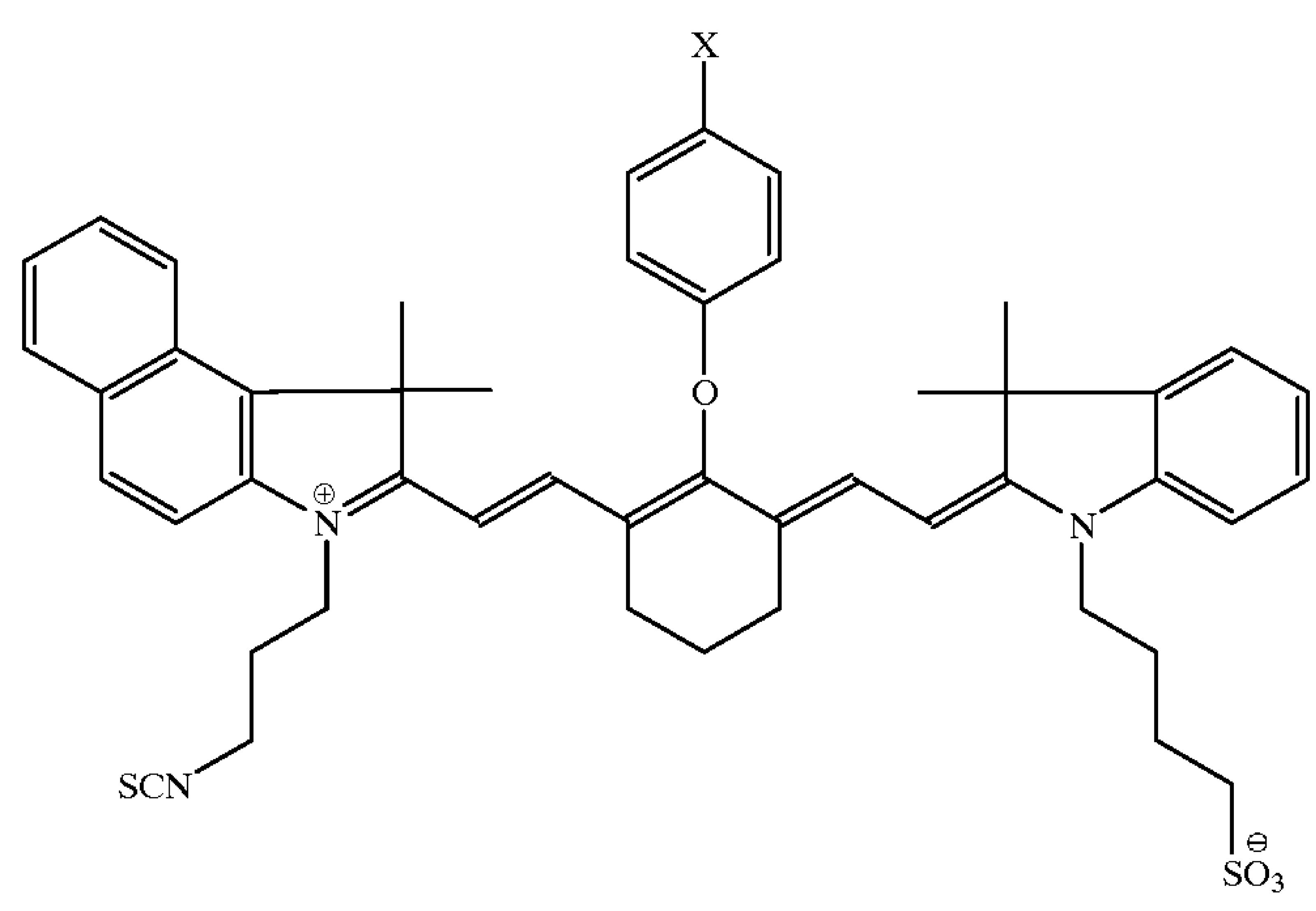
(2) —CH_2—CH_2—CH_2—OH for an absorbance wavelength of 780 nanometers;

(3) —CH_2—CH_2—CH_2—CH_2—CH_2—CH_2—OH for an absorbance wavelength of 745 nanometers; and

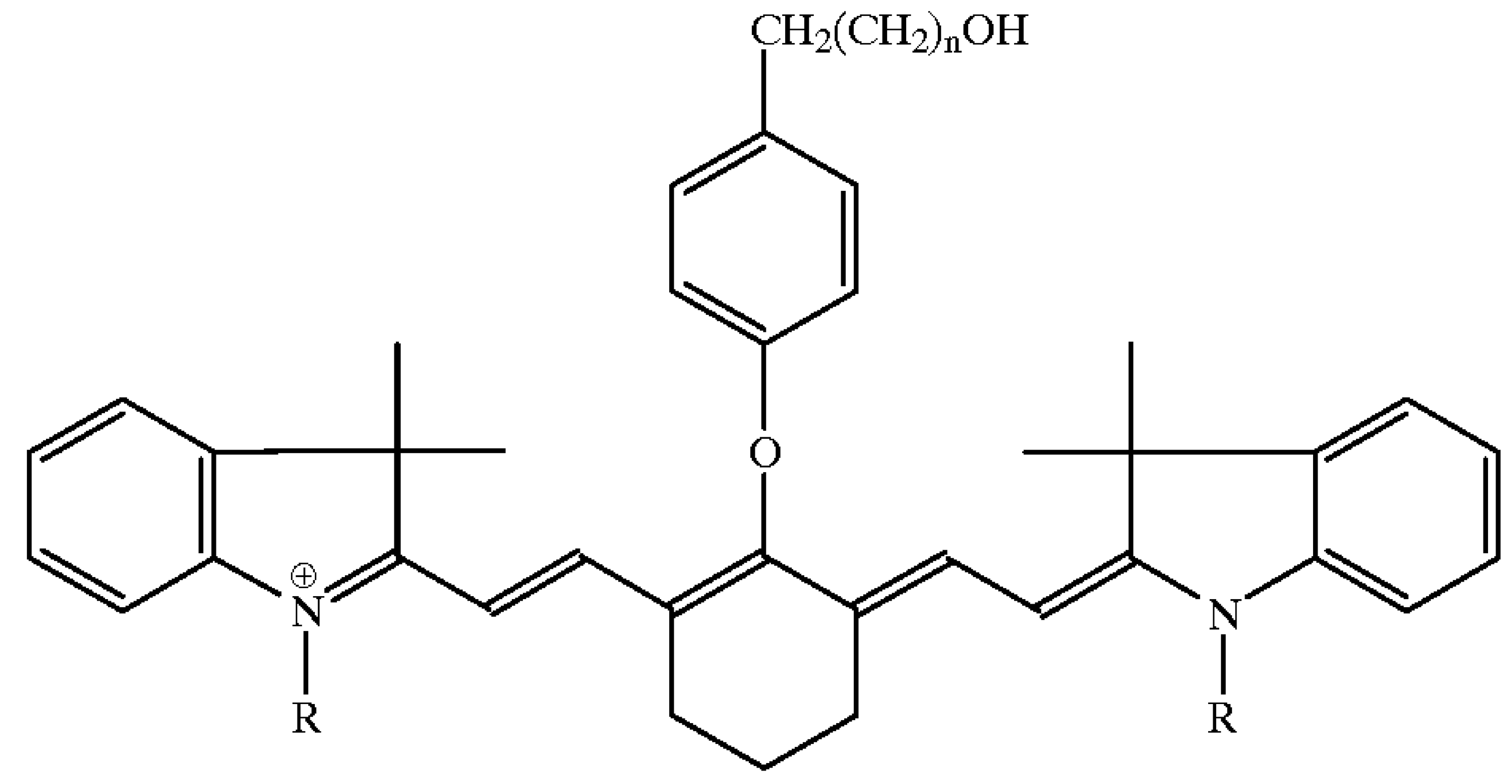
(4) —CH_2—CH_2—O—CH_2—CH_2—O—CH_2—CH_2—OH for an absorbance wavelength of 790 nanometers.

In FIG. **4**, there is shown an embodiment E10 of sequencing system having a central system 120, a plurality of remote stations, two of which are shown at **122**A and **122**B and a DNA fluorescent marking system **121**. The DNA fluorescent marking system **121** includes means for labeling identical strands of DNA

FORMULA 4

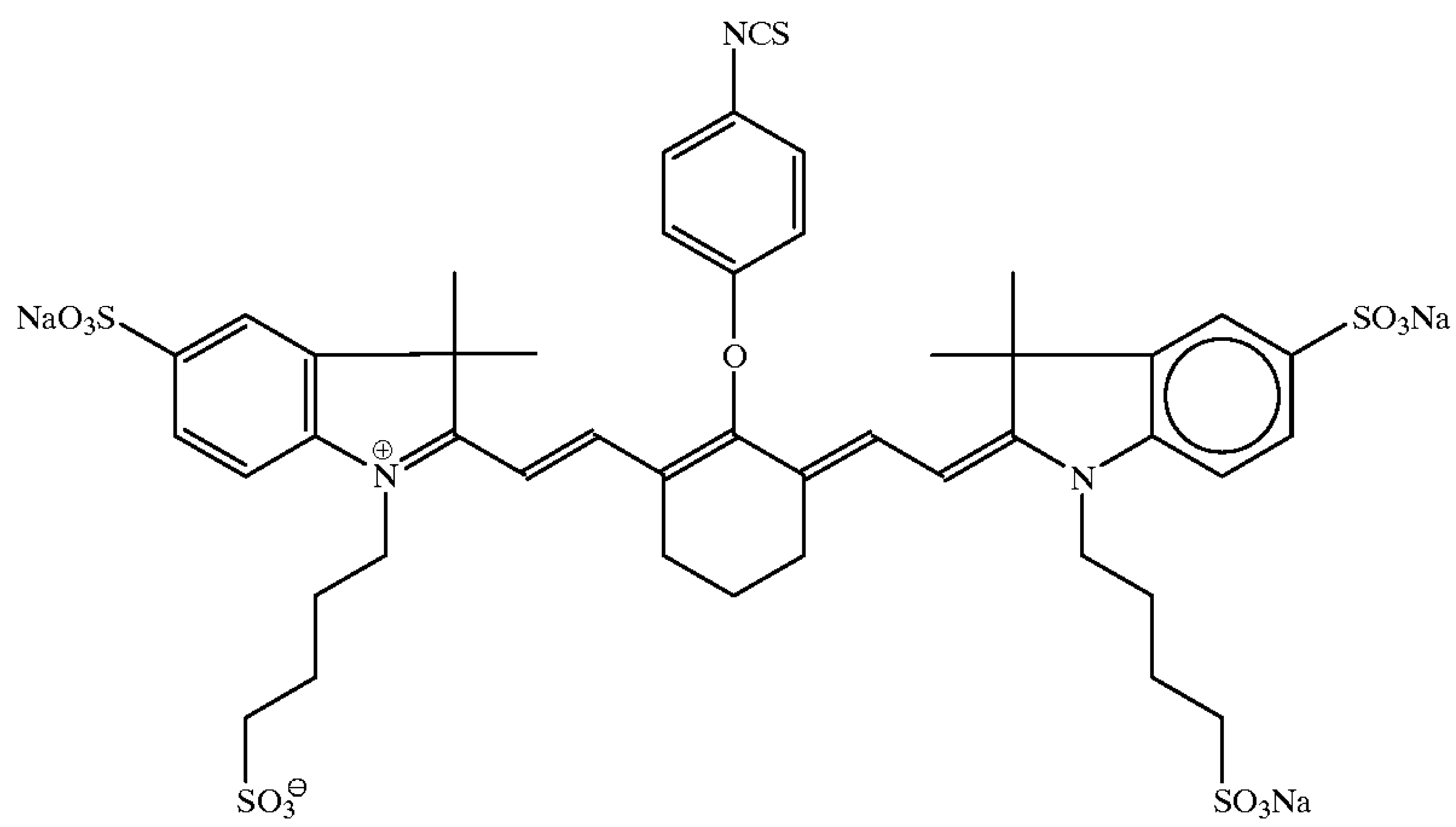


FORMULA 5

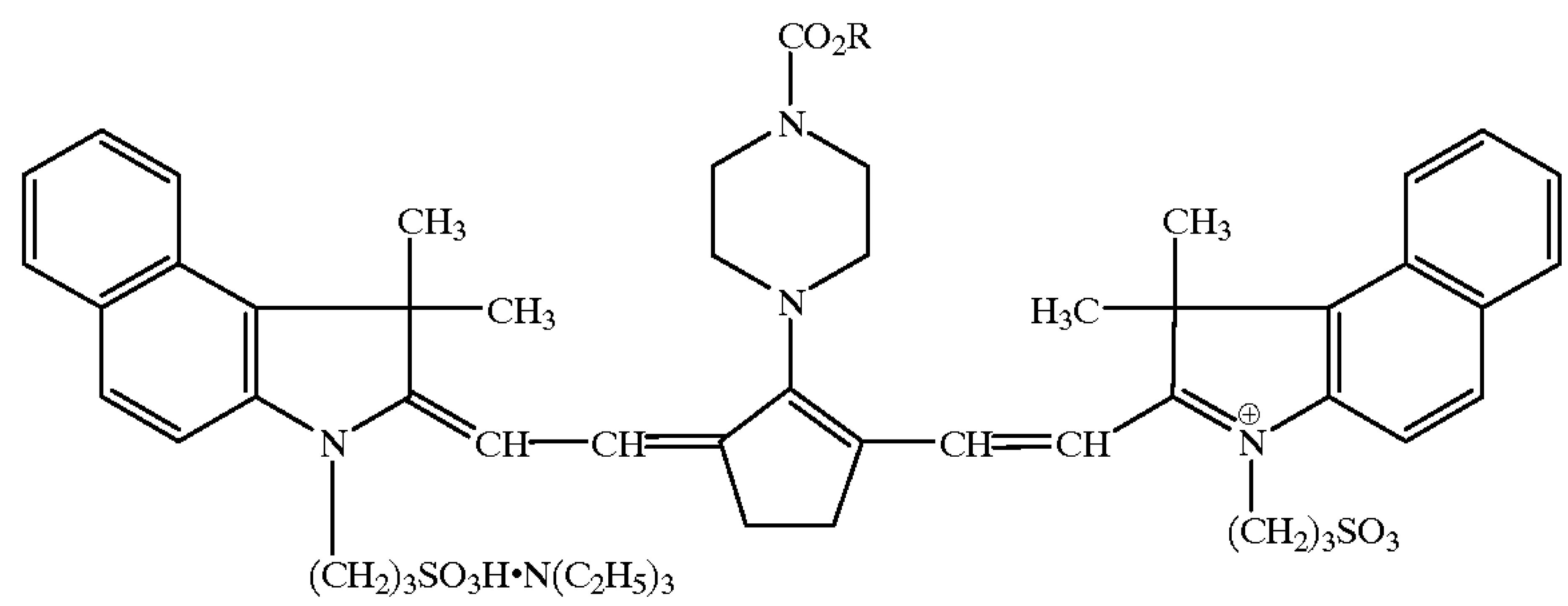


-continued

FORMULA 6



FORMULA 7



and a DNA preparation system. In this preparation process, strands are separated into four aliquots. The strands in a given aliquot are synthesized to any base belonging to one or more of the four base types, which are adenine, guanine, cytosine and thymine (hereinafter A, G, C and T). The adenine-, guanine-, cytosine- and thymine-terminated strands are then electrophoresed for separation. The rate of electrophoresis indicates the DNA sequence.

The fluorescent markers are attached to the identical strands of more than 100 bases in a container. The fluorescent markers may be attached to DNA primer molecules or to deoxynucleotide triphosphates used in the synthesis of the DNA strands or to dideoxynucleotide triphosphates which terminate synthesis of the DNA strands or to universal terminators. Single or multiple fluorescent markers may be attached to the DNA fragments. They must be of such a size and have such chemical characteristics as to not obscure the normal differences in the mobilities between the different fragments due to terminations at different ones of the adenine, guanine, cytosine and thymine bases and be able to be easily detected.

The DNA fluorescent marking system **121** communicates with the central system **120** as well as the remote stations **122**A and **122**B. The central system **120** includes a separating system and a detection and processing system to separate the strands by length with each fragment being terminated at a different one of the A, T, G and C groups. The separating system, which sequences strands by length, communicates with the detection and processing system which analyzes the fragments by comparison of the progress of each band of DNA fragments along the gel with the other bands to derive information about the sequence of the DNA.

The separating system continuously sequences strands of DNA, and for this purpose, the preferred embodiment includes at least four electrophoresis channels, each adapted to receive fluorescently labeled DNA strands having at one end a universal terminator substituted for a base of a given type. Each of the channels has a gel path and electrical field across it identical in its characteristics to the gel path of the other channels and electrical fields across the other channels. The bands are detected in a manner that indicates their mobility in the gel to indicate the sequence of the A, G, C and T strands of different lengths.

The detection and processing system includes a scanning apparatus having a light source, such as a laser diode or light-emitting diode or other suitable source that emits light in the optimum absorbance spectrum of the marker. The light may be split by the use of fiber. In the preferred embodiment, the light source is a laser diode that irradiates the channels with far red or near infrared light having a wavelength that matches the absorbance region of the marker. The detector includes a light sensor that is preferably an avalanche photodiode that is sensitive to the far red or near infrared light emission of the marker. It may include a filtering system having a pass band suitable for passing selectively the optimum emission of the fluorescent marker to the light sensor.

The photodiode, photomultiplier or other light sensor selectively detects the fluorescence using techniques which enhance the signal/noise ratio. One technique is to modulate the laser source by pulsing the electrical current driving the laser and detect light in sequence with the emitted light by connecting the output signal from the sensor with a lock-in amplifier that is sequenced with the pulsed laser light. Another technique is the use of laser pulses which are less than five nanoseconds time duration, with detection in a time window. The length of such window and its delay from the pulse are optimized to discriminate against background fluorescence as well as scattered laser light.

Another technique is the use of sinusoidally modulated laser pulses combined with phase modulation discrimination.

To determine the sequence of strands, the processing system includes means for correlation between the channel in which the fluorescent light is detected with the time of detection and means for indicating: (1) which of the base types, A, G, C or T, the termination is associated with; and (2) the time sequence of separation of each strand in each channel of the electrophoresis gel.

To use the apparatus to sequence DNA strands, identical DNA strands are normally formed of a length greater than 100 bases. In one embodiment, the strands are marked by a suitable marker. The strands are divided into four aliquots and the strands within each aliquot are synthesized to any base belonging to a specific base type. These four aliquots are then electrophoresed through identical channels to separate strands so that the shorter strands are resolved towards the end of the gel prior to resolution of the longer strands, which still are near the entrance end of the gel. In another embodiment, the strands are divided into four aliquots and synthesized to a given base, with marking ocurring during termination of synthesis. The same marker may be used for all four aliquots and separation may be performed as described above or a different marker may be used for each different termination group of the A, T, C and G groups so as to process in a single channel for a complete sequence. This occurs in a continuous process so a substantial number of different length strands may be resolved in a relatively short gel. This methodology takes advantage of time-resolved bands, as opposed to the limitations of spatial-resolved bands.

The gel size, electric field and DNA mobilities are such that the more mobile bands are fully resolved while the less mobile bands are yet unresolved in a continuous process such that at least ten percent of the bands have been resolved by electrophoresis in the gel while the less mobile bands which are near the entrance end of the gel are not fully resolved. These less mobile bands become resolved little by little over time in a continuous fashion without interruption of the movement of these bands through the gel. The markers are detected by transmitting far red or near infrared light to fluorescently marked DNA strands which may be at the same far red or near infrared wavelength or at different far red or near infrared wavelengths depending on the embodiment of separation technique.

The remote stations **122**A and **122**B each are able to perform the sequencing but some portions of data processing can only be performed by the central station **120**. It may supply data to the remote stations, such as **122**A and **122**B, to which it is electrically connected and receive data from them. With this arrangement, the central sequencing system **120** may cooperate with one or more of the remote stations, such as **122**A and **122**B, for increased capability such as increased number of channels. Each unit may control the parameters used in sequencing, such as the electrophoresis potential or the like.

In FIG. **5**, there is shown a simplified view of the remote station **122**A having a cabinet housing **130**, a front cover **132**, a liquid crystal display readout **134**, a high voltage warning light **136** and a plurality of function keys **138**. In FIG. **5**, the remote station **122**A is shown closed. However, the front cover **132** may be removed to expose an electrophoresis section. The potential applied across the gel may be set and different data readouts may be selected either from the analysis provided within the central system **120** (FIG. **4**) or values from within the remote station **122**A using the function key pad **138** and the selected data displayed on the liquid crystal display readout **134** prior to and/or after selection.

In FIG. **6**, there is shown a sectional view of a portion of the remote station **122**A taken through section lines **6**–**6** of FIG. **5** having an electrophoresis section **140**, a scanning section **142**, an electrophoresis power supply **144**, a system power supply section **144**A, an analog board **146** and a digital board **148**. The electrophoresis section **140** is positioned near the front of the cabinet and a portion of it is adapted to be scanned by the scanning section **142** in cooperation with circuitry on the analog board **146** and the digital board **148**. All of the apparatus are electrically connected to the power supply section **144**A for such operation.

To separate different DNA fragments into bands, the electrophoresis section **140** includes a gel sandwich **150**, an upper buffer assembly **152**, a support assembly **154**, and a lower buffer assembly **151** positioned to enclose the bottom of the gel sandwich **150**. In the embodiment of FIG. **6**, the gel sandwich **150** is held vertically and its temperature is controlled during operation. Bands are separated by applying voltage to the upper buffer assembly **152** and lower buffer assembly **151** and scanned by the scanning section **142**.

To support the gel sandwich **150**, the support assembly **154** includes a pair of upper side brackets and lower side brackets **160** and **162** (only one of each pair being shown in FIG. **6**), an apparatus support plate **168**, a temperature control heating plate **164** and a plastic spacer, shown at **166**A–**166**C, in FIG. **6**. The entire structure is supported on the apparatus support plate **168** which mounts the upper and lower side brackets **160** and **162**.

The upper and lower side brackets **160** and **162** are each shaped to receive a pin such as **161** and **167** (FIG. **9**) extending from a gel sandwich such as the gel sandwich **150** and thus hold the gel sandwich in place on one side in juxtaposition with the scanning section **142**. The pin **167** (FIG. **9**) on the side of the sandwich opposite to the pin **161** (FIG. **9**) fits into a corresponding one of two brackets **163** and **160** (FIG. **9**) so that the gel sandwich can be hooked in place. The other two brackets **165** and **162** are positioned to receive the pins of other length gel sandwiches with the lower bracket **162** receiving pins of shorter vertical length sandwiches than the upper bracket **160**. Even longer gel sandwiches can be mounted by substituting a longer heating plate for the heating plate shown at **164**.

The spacers as shown as **166**A–**166**C space the temperature control heating plate **164** from the apparatus support plate **168** and maintain it at a constant selected temperature above ambient temperature. In the preferred embodiment, the temperature is maintained at 45–50 degrees Centigrade and should be maintained in a range of 30 degrees to 80 degrees.

The scanning section **142** includes a laser diode assembly (not shown in FIG. **6**), a microscope assembly **172**, a photodiode section **174** and a scanner mounting section **176**. The laser diode assembly (not shown in FIG. **6**) is positioned at an angle to an opening in the apparatus support plate **168** and the heating plate **164** so that light impinges on the gel sandwich **150** to cause fluorescence with minimum reflection back through the microscope assembly **172**.

To receive the fluorescent light, the microscope assembly **172** is focused on the gel sandwich **150** and transmits fluorescent light emitted therefrom into the photodiode section **174** which converts it to electrical signals for transmission to and processing by the analog and digital boards **146** and **148** which may provide further analysis of data. The scanning section **142** moves along a slot in the apparatus support plate **168** which is mounted to the scanner mounting section **176** during this operation in order to scan across the columns in the gel sandwich **150**.

The scanner mounting section **176** includes a mounting plate **180**, a bearing plate **182**, a stepping motor **184**, a slidable support **186** and a belt and pully arrangement **185**, **188**A and **188**B. The mounting plate **180** is movably mounted to the apparatus support plate **168** through a frame member and supports the elongated bearing plate **182**, the stepping motor **184** and two pulleys **188**A and **188**B. The elongated bearing plate **182** extends the length of the gel sandwich **150**.

To permit motion of the laser diode assembly (not shown) and microscope assembly **172** with respect to the gel sandwich **150**, the slidable support **186** supports the microscope assembly **172** and laser diode assembly and slidably rests upon the bearing plate **182**. An output shaft **183** of the stepping motor **184** drives a pulley **188**B through pulley **188**, belt **185**, and pulley **188**A and the pulley **188**B drives a belt (not shown) that is clamped to the slidable support **186** to move it the length of the gel sandwich **150** during scanning by the laser diode and microscope assembly **172** which rest upon it. The stepping motor **184** under the control of circuitry in the digital board **148** moves the pulley **188**B to move the belt (not shown) and thus cause scanning across the gel sandwich **150**.

As shown in this view, the electrophoresis power supply **144** is electrically connected to buffer in the upper buffer assembly **152** through an electrical connector **194** and to the lower buffer assembly **151** through a connector not shown in FIG. **6**.

The upper buffer assembly **152** includes walls **197** forming a container to hold a buffer solution **195** and a cover **199** formed with a lip to fit over the walls **197** from the top and containing a downwardly extending flat member spaced away from the side walls and holding a conductor **211**. The conductor **211** is electrically connected to the source of power through connector **194** which is mounted to the top of the cover **199** to permit electrical energization of the buffer solution **195**.

The bottom buffer assembly **151** includes enclosed walls **201** defining a container for holding a buffer solution **203** and a cover **205** closing the container **201** and having a downwardly extending portion **213** extending into the buffer solution **203** for supporting a conductor **207** for applying energy to the bottom buffer solution **203**. The gel sandwich **150** extends downwardly into the buffer solution **203** and upwardly into the buffer solution **195** to permit the electrical contact for electrophoresis. An "O" ring **197**B provides a seal for the upper buffer assembly **152** so that the buffer solution **195** does not empty out of the upper buffer assembly **152**.

In FIG. **7**, there is shown a sectional view taken through lines **7**–**7** of FIG. **5** showing a portion of the electrophoresis section **140**, a portion of the scanning section **142** (indicated twice in FIG. **7** for clarity) and the electrophoresis power supply section **144** (FIG. **6**) mounted together to illustrate, from a top view, the arrangement of the apparatus support plate **168**, the heater plate **164**, the gel sandwich **150**, a laser diode assembly **170**, a microscope assembly **172** and a photodiode assembly **174**. The heater plate **164** and apparatus support plate **168** have slots running in a horizontal direction orthogonal to the lanes of DNA in the electrophoresis section **140** sized to receive the ends of a laser diode assembly **170** and the microscope assembly **172** for scanning thereof.

To cooperate with the separation and scanning of DNA bands, the gel sandwich **150** includes a front glass plate **200**, a gel section **202** and a rear glass plate **204** mounted in contact with the heater plate **164** and having a section exposed for scanning by the laser diode assembly **170** and the microscope assembly **172**. The rear glass plate **204** contacts the heater plate **164** and is separated from the front glass plate **200** by the gel section **202** within which DNA separation takes place. The front and rear glass plates **200** and **204** may be any type of glass but are preferably soda lime which has low fluorescence in the far red and near infrared regions and is prepared by a process that provides optically flat surfaces without grinding.

To transmit light to the gel sandwich **150**, the laser diode assembly **170** includes a housing **210**, a focusing lens **212**, a narrow band pass filter **214**, a collimating lens **216** and a laser diode **218**. The laser diode **218** emits far red or near infrared light which is collimated by the laser collimating lens **216** and filtered through the narrow band pass filter **214**. This light is focused by the focusing lens **212** onto the gel sandwich **150**. Preferably, the point of focus on the gel section **202** of the gel sandwich **150** lies along or near the central longitudinal axis of the microscope assembly **172** and the photodiode assembly **174**.

The thickness of the glass plates and the gel, the position of the laser and microscope assembly and thus the angle of incidence and angle of reflection of the light from the laser and to the microscope assembly **172** are chosen, taking into consideration the refractive index of the gel and glass and the thickness of the glass plates and the gel, so that the light from the laser is maximally transmitted to the gel. The light from the laser is not directly reflected back because the angle of incidence to normal is equal to the Brewster's angle at the first interface and is such as to impinge on the markers with full intensity after refraction but not be reflected by the first surface of the gel sandwich **150** into the microscope assembly **172** and the microscope assembly **172** views those markers that fluoresce in its line of sight.

To maintain temperature control over the laser diode, the housing **210**: (a) is coupled to a heat sink through a thermal electric cooler **220**, and (b) encloses the focusing lens **212**, narrow band pass filter **214**, collimating lens **216** and laser diode **218**; and (c) accommodates the electrical leads for the diode.

To receive and focus light emitted by fluorescent markers from the gel section **202** in response to the light from the laser diode assembly **170**, the microscope assembly **172** includes a collection lens **230**, a housing **232**, and a focusing motor. The microscope assembly **172** is adapted to be positioned with its longitudinal axis centered on the collection lens **230** and aligned with the photodiode assembly **174** to which it is connected. For this purpose, the housing **232** includes a central passageway in which are located one or more optical filters (not shown) with a pass band matching the emission fluorescence of the marked DNA strands. With this arrangement, the collection lens **230** receives light from the fluorescent material within the gel section **202** and collimates the collected light for optical filtering and then transmission to the photodiode assembly **174**.

To generate electrical signals representing the detected fluorescence, the photodiode assembly **174** includes a housing **240** having within it, as the principal elements of the light sensor, an inlet window **242**, a focusing lens **244**, a sapphire window **246** and an avalanche photodiode **248**. To support the avalanche photodiode **248**, a detector mounting plate **250** is mounted within the housing **240** to support a plate upon which the avalanche photodiode **248** is mounted. The inlet window **242** fits within the housing **240** to receive light along the longitudinal axis of the photodiode assembly **174** from the microscope assembly **172**.

Within the housing **240** of the photodiode assembly **174**, the sapphire window **246** and avalanche photodiode **248** are aligned along the common axis of the microscope assembly **172** and the photodiode assembly **174**. The focusing lens **244** focuses light transmitted by the microscope assembly **172** onto a small spot on the avalanche photodiode **248** for conversion to electrical signals. A thermoelectric cooler **252** utilizing the Peltier effect is mounted adjacent to the detector mounting plate **250** to maintain a relatively cool temperature suitable for proper operation of the avalanche photodiode **248**.

As best shown in this view, the stepping motor **184** rotates the belt **185** to turn the pulley **188**A, which, in turn, rotates pulley **188**B. The pulley **188**B includes a belt **177** extending between it and an idler pulley **179** and is attached at one location to the slideable support **186** (FIG. **6**) to move the scanning microscope and laser lengthwise along the gel sandwich **150** for scanning purposes. The motor **184**, by moving the carriage back and forth accomplishes scanning of the gel sandwich **150**.

In FIG. **8**, there is shown a fragmentary perspective view of the gel sandwich **150** and the upper buffer assembly **152** mounted to each other showing the outer glass plate **200** cut away from the rear glass plate **204** to expose the gel section **202** to buffer solution within the upper buffer assembly **152**. With this arrangement, DNA samples may be pipetted between the glass plates **200** and **204** and moved downwardly by electrophoresis beyond the upper buffer assembly **152** and through the gel sandwich **150** to the bottom buffer (not shown in FIG. **8**).

In FIG. **9**, there is shown a broken away view of the gel sandwich **150** illustrating the upper buffer assembly **152** and the lower buffer assembly **151** connected to it at each end. As shown in this view, the cover **199** includes a connecting post **214** which receives the elongated flexible conductor **211** for connection to the downwardly extending portion of the cover **199** into the buffer compartment. This flexible conductor has sufficient length to accommodate different lengths of gel sandwiches that cause the upper buffer assembly **152** to be at different elevations. In another embodiment, the length of conductor **211** is the same for the different lengths of gel sandwiches in that this cover **199** is raised or lowered depending on the gel length. To accomodate electrical connection in this embodiment, either the connecting post **214** mates with one of a series of mounting connectors located at different vertical positions on the apparatus support plate **168** (FIG. **6**) or the connecting post **214** mates with a flexible extension cable that electrically connects the connecting post **214** with a mounting connector.

As best shown in this view, a plurality of side brackets **160**, **163**, **165** and **169** are mounted to the apparatus support plate **168** (FIG. **6**) to receive pins **161** and **167** extending from the sides of the gel sandwich **150** to support the gel sandwich in place. The pins **161** and **167** extend from opposite sides of the gel sandwich at the same elevation and the brackets **160**, **163**, **165** and **169** are mounted in pairs to the apparatus support plate **168** (FIG. **6**) with each pair being at a different elevation and each bracket of a pair of brackets being positioned on the opposite side of the gel sandwich from the other bracket of the same pair to support different sizes of gel sandwichs at a location that provides balance to them.

The upper and lower side brackets **160** and **162** on one side of the gel sandwich and the upper and lower side brackets **163** and **165** on the opposite side are each shaped to receive a pin such as **161** and **167** (FIG. **9**) extending from the gel sandwich **150** and thus hold the gel sandwich in place on one side in juxtaposition with the scanning section **142** (FIGS. **6** and **7**). The pin **167** on the side of the sandwich opposite to the pin **161** fits into a corresponding one of the two brackets **163** and **165** so that the gel sandwich can be hooked in place. For longer gel sandwiches, the pin **167** fits into bracket **163** while for shorter gel sandwiches the pin **167** fits into bracket **165**. The other of the two brackets **160** and **169** are positioned to receive the pin located on the opposite side of the gel sandwich such that the lower bracket **169** receives the pin of shorter vertical length gel sandwiches and the upper bracket **160** receives the pin of longer vertical length gel sandwiches. Even longer gel sandwiches can be mounted by substituting a longer heater plate for the heater plate shown at **164** (FIG. **6**). As best shown in this view, recesses **231** extend downwardly into the gel to receive DNA sample from a pipette and thus form channels for electrophoresing.

To form an electrical connection through the gel sandwich **150** from the upper buffer assembly **152** to the lower buffer assembly **151**, a connecting post **218** is connected to the cover **205** of the lower buffer assembly **151** for receiving the conductor **207** (FIG. **6**) which extends downwardly to the downwardly extended plate **213** and into the buffer solution.

In FIG. **10**, there is shown a block diagram of the circuitry used to control the remote station **122**A of the embodiment of FIG. **5** having a control, correlation and readout section **250**, a scanner drive **176**, the motor assembly **184** for moving the scanner drive **176**, the sensing configuration **252** and the focusing motor assembly and pivot motor assemby controls **300** and **302** respectively.

The sensing configuration **252** includes the laser diode assembly **170** and the photodiode assembly **174** which receives signals, removes some noise, and transmits the signals for display and readout in the control, correlation and readout section **250**. At the same time, the scanner drive **176** and motor for the scanner drive **184** receive signals from the control, correlation and readout section **250** to control the motion of the sensor back and forth across the gel sandwich. This overall configuration is not part of the invention of this application except insofar as it cooperates with the sensing configuration **252** to scan the DNA and determine its sequence in accordance with the embodiments of FIGS. **5**–**10**.

To drive the laser diode assembly **170** and the microscope assembly **172** (FIGS. **6** & **7**) and the photodiode assembly **174** from position to position, the motor assembly **184** includes a stepping motor **254** and a motor driver **256**. The motor drive **256** receives signals from the control correlation and readout section **250** and actuates the stepping motor **254** to drive the scanner drive **176**. The scanner drive **176** is mechanically coupled to the stepping motor **254** through a belt and pulley arrangement for movement back and forth to irradiate and sense the electrophoresis channels on the gel sandwich **150** (FIG. **6**). The stepping motor **254** and motor driver **256** are conventional and not themselves part of the invention.

To correlate the scan with received signals and provide a display of them, the control, correlation and readout system **250** includes a computer **260** which may be any standard microprocessor, a television display or cathode ray tube display **262** and a printer **264** for displaying and printing the results of the scans. Data, after being processed in sensing configuration **252** is supplied to the computer **260** for correlation with the position of the scanner drive **176** as controlled by the computer **260** and display **262**.

To sense data, the sensing configuration **252** includes in addition to the laser diode assembly **170** and the photodiode assembly **174**, a chopper circuit **270**, a sensor power supply **272**, a preamplifier **274**, a lock-in amplifier **276**, a 6-pole filter **278**, a 12-bit analogue to digital converter interface circuit **280** and a laser power supply **282**. The photodiode assembly **174** receives light from the laser diode assembly **170** after it impinges upon the gel sandwich **150** (FIG. **6**) and transmits electrical signals through preamplifier **274** to the lock-in amplifier **276**. The photodiode assembly **174** receives signals from the sensor power supply **272**. The chopper circuit **270** provides pulses at synchronized frequencies to the lock-in amplifier **276**.

The laser diode assembly **170** receives power from the power supply **282** which is controlled by the chopper circuit **270** so that the signal from the laser diode assembly **170** is in synchronism with the signal applied to the lock-in amplifier **276** so that the output from the lock-in amplifier **276** to the 6-pole filter **278** discriminates against unwanted signal frequencies. This signal is converted to a digital signal in the 12-bit analogue to digital converter **280** which serves as an interface to the computer **260**.

To maintain optical focus, the computer **260** is electrically connected to the pivot motor assembly **302** and the focusing motor assembly **300**, which motor assemblies are able to adjust the microscope assembly **172** (FIGS. **6** and **7**) to focus it and to adjust the location of the microscope with respect to the gel sandwich **150** by pivoting the support bed **180** (FIGS. **7** and **11**). As will be better explained hereinafter, this permits a number of different focusing arrangements such as one in which the microscope is periodically refocused during an individual scan across the gel to maintain focus and one in which the distance between the gel sandwich and the path of travel of the microscope is adjusted with respect to each other so that during movement of the microscope as it scans, the focal point is maintained on the gel even though there was originally some non parallelism between the gel and the travel path of the microscope along the course of a scan. Of course both motors may be controlled simultaneously as better explained hereinafter.

In FIG. **11**, there is shown a fragmentary, exploded top perspective view of another embodiment of scanning section **142A** substantially the same as the scanning section **142** of FIGS. **6** and **7** in which the identical parts are indicated by the same reference numerals in each embodiment. As shown in this view, the scanning section **142A** includes three motor assemblies, the stepping motor assembly (scan motor assembly) **184**, the focusing motor **300** and a mounting plate pivot motor assembly **302** and a pivot assembly **304**. The mounting plate pivot motor assembly **302** is only in the embodiment of scanning assembly **142A** but the focusing motor **300** and stepping motor assembly **184** are in both the embodiment of scanning section **142** and the embodiment **142A**. The motor assembly **184** operates in the same manner in both embodiments to drive the output shaft **183** (FIGS. **6** and **12**) which in turn drives the slidable support **186** (FIG. **6**) on the bearing plate **182** (FIGS. **6** and **7**) through the belt **185** (FIGS. **6** and **12**) and toothed belt **177**.

The focusing motor assembly **300** is mounted for movement with the microscope assembly **172** and photodiode assembly **174** to focus the microscope assembly **172** directly into the gel to receive light directly from the fluorescent markers therein. This focusing may be done manually or automatically by focusing at points where there is no fluorescent emission from DNA markers in the gel. This is done by sensing the fluorescence of the two glass plates which have relatively high emission and causing the focusing to be between the two high emission glass plates and within the lower emission gel.

The pivot motor assembly **302** cooperates with the pivot assembly **304** to adjust the angle between the gel sandwich **150** (FIG. **6**) and the mounting plate **180** so that the focus of the microscope assembly **172** can be set and the focal point remain within the gel section as the microscope assembly **172** moves in a horizontal scanning direction across the gel sandwich **150** (FIG. **6**). The pivot motor assembly **302** cooperates in the focusing operation so that the microscope is focused at one point at one end of the gel sandwich **150** and then the microscope assembly **172** scans across to another widely separated point without changing the focus of the microscope lens.

After the microscope assembly **172** has moved to a new location, the pivot motor assembly **302** then moves the support bed **180** about the pivot point **305** with the motion being in a horizontal plane to adjust the angle in the vertical plane of the support bed **180** and gel sandwich **150** with respect to each other so that the plane of the gel sandwich **150** and the scan direction are parallel. The focus motor **300** then refocuses the microscope assembly **172** so that the focus point is the same at both extremes of a scan, thus permitting a continuous scan without the need to dynamically refocus the microscope. Of course either the gel sandwich or support for the microscope or both can be adjusted with minor modification of the equipment.

To permit adjustment of the horizontal scanning path of the microscope assembly **172**, the pivot assembly **304** in the preferred embodiment includes an opening or eyelet at pivot point **305** with the pivot point **305** having a vertical axis perpendicular to the support plate **180**, a cylindrical vertically oriented pivot pin **310** and a mounting housing **311** rigidly mounted to the pivot pin **310**. The pivot pin **310** fits rotatably within the support bed **180** and the support bed **180** is movably bolted to the housing **311**. The bolts (not shown in FIG. **11**) have shanks that extend loosely through the openings **313** and **315** to permit movement between the support plate **180** and housing **311** to permit pivoting of the support bed **180** with respect to the housing **311**. The housing **311**, which is mounted at end **317**, and the corresponding end of a support member **306** are mounted to the apparatus support plate **168** (FIG. **6**) to movably support the two ends of the support bed **180** on the DNA sequencer frame.

To provide pivoting, the pivot motor assembly **302** includes the motor output shaft **319**, biasing member **321** and support member **306** so that rotation of the motor **303** in one direction causes the motor output shaft **319** to push against the vertical apparatus support plate **168** (FIG. **6**) to which it is movably mounted at one end to increase the angle, and rotation in the other direction pulls it to reduce the angle or releases it to be pulled by a biasing member **321** to move the bed forwardly to the plate **168** and decrease the angle. The support member **306** is mounted to the support plate **180** by bolts (not shown in FIG. **11**) having their shanks fitting through slots similar to **313** and **315** that are large enough to permit pivoting. The support member **306** is mounted to vertical apparatus support plate **168** so that the support member **306** is supported to the frame of the DNA sequencer.

In FIG. **12**, there is shown a bottom perspective view of the support plate **180** showing a mounting support means **308**, shaft **183**, pivot hole **305** and bolt holes **313** and **315**, the motor assembly **184**, the pivot and the pivot motor assembly **302** illustrating the manner in which the pivot pin is mounted to the frame to permit movement of the support plate **180** by the pivot motor assembly **302**.

In the embodiment of FIGS. **11** and **12** the angle of the support plate **180** is changed to cause the microscope to remain focused during a scan. In another embodiment, the angle of the support plate **180** is not changed but the microscope is refocused at a number of points during a horizontal scan to maintain the focus point within the gel. This method has certain inertia problems which slow down the scan or decrease its precision because of the larger number of times the assembly must start or stop.

In FIG. **13**, there is shown a general block diagram **320** of a control program permitting the computer to control the focusing of the microscope during a scan so that the focal point remains within the gel of the gel sandwich. This program includes the general step **322** of obtaining focus points such as a single right and left point or a number of points and the step **324** of adjusting for scan depth and performing the scan.

In one embodiment, the focus points are in the gel away from the channels having DNA bands on both the right and left side of a horizontal scan and in another embodiment, it is at a plurality of points along a scan between channels having bands as well as near the ends of the scans. In another embodiment, the focusing is performed prior to the DNA bands being electrophoresced so that it is not necessary to select particular scan locations for the purpose of avoiding such DNA bands. In the former embodiment, the microscope is focused within the gel at one side, in which side there is the pivot point **305** (FIG. **11**) in the scan support plate **180**, and then at the second point. The microscope is moved to the second point without changing the scan. When the microscope is at the second point, the support plate **180** is moved so that, without adjusting the microscope further, the gel sandwich and support bed are altered in a parallel position. Then the microscope is refocused such that the focal point is within the gel for both locations, thus enabling a continuous scan thereafter which can proceed without dynamic focusing.

In the latter embodiment, as a scan is performed, the microscope assembly **172** refocuses at a number of points, preferably refocusing the lens by means of the focusing motor assembly, although the focusing could be done by adjusting the position of the support bed and gel sandwich with respect to each other or both focusing the lens and the position of the support bed and gel plate with respect to each other.

In FIG. **14**, there is shown a block diagram of a subsequence of substeps within the step **322** for obtaining reference focal points including: (1) the substep **324** of beginning the program; (2) the substep **326** of moving the microscope to the left side of the apparatus (the zero position) and turning the laser on; (3) the substep **328** of backing the focus motor up 0.008 inches in 16 motor steps (the distance from microscope to gel is now 0.008 inches beyond the starting point at the gel sandwich); (4) the substep **330** of causing the focusing motor assembly to move forward one step, measuring the image signal one hundred times and obtaining the average of it; (5) the substep **332** of counting the steps the motor moves forward; (6) the substep **333** of deciding whether the count equals 32 motions forward or not, returning to the step **330** if it does not and if it does, the decision **332** to move to step **334**; (7) the substep **334** of causing the focus motor to move back to its starting point; (8) the substep **336** of moving to a new position on the other side of the apparatus (right side in the preferred embodiment) at specified points such as one inch increments up to six inches along the scan and repeating steps **330** through **336** for each incremental location; and (9) followed by the decision step **336** of moving the scan back to its original zero point at end step **339**.

With this arrangement, the microscope is moved to a starting point and focused at a number of locations for that particular starting point, such locations including the gel within them as well as portions of the glass supporting plates. Data is taken at each location a multiple number of times and averaged for precision, with this data being stored. The microscope is then moved in a horizontal scan operation to another point and the process repeated so that at least two data points are obtained and stored in the memory of the computer. These data points permit the position of the microscope and gel to be adjusted with respect to each other to maintain focus between them. It may be necessary to calculate the amount of pivoting of the microscope support and gel about a pivot point if a measurement is not directly at the pivot point.

In FIG. **15**, there is shown a block diagram illustrating a subsequence within the step **324** (FIG. **13**) of adjusting for scan depth and performing a scan including: (1) the substep **340** of determining where the minimum light emission occurred at different points; (2) the substep **342** of having the focus and pivot points moved so that the microscope is focused at the minimum fluorescence point at all of the locations; (3) the substep **344** of turning off the laser, moving the microscope to the zero position and resuming scanning with the laser turned back on if desired; and (4) the substep **346** of terminating the scan.

In FIG. **16**, there is shown a block diagram of a program **350** illustrating a dynamic mode of scanning including: (1) the substep **352** of determining the minimum reading locations for left and right sides; (2) the substep **354** of determining the position along an equidistant straight line for at least eight points; (3) the substep **356** of moving the microscope to the zero position; (4) followed by the step **358** of scanning one section; (5) the step **360** of adjusting the focus again; (6) the step **362** of counting the adjustment; (7) the decision step **364** of determining if it is eight counts, and if not, returning to step **358** and repeating, and if it is, ending the scan; and (8) substep **366** of ending the dynamic focusing operation. With this arrangement, the microscope is refocused eight times in a scan operation.

In FIG. **17**, there is shown a program **370** for controlling the scan comprising: (1) the step **372** of beginning the scan; (2) the step **374** in which the customer uses the host computer or scanner keyboard to start scanning; (3) the step **375** in which the scanner software initializes DMA (direct memory access) pointers and initializes a final position in the motor control integrated circuit; (4) the step **376** in which the scanning software tells the motor control integrated circuit to begin moving the microscope; (5) the step **378** in which a traverse of the microscope each 0.00048 inches causes the analog-to-digital converter to take one measurement and store that reading in memory; (6) the step **380** in which the scanner microprocessor is interrupted to say that a run is completed when the motor control integrated circuit is done moving a single one-way trip, either left or right; (7) the step **382** of processing data and sending it to the host computer while the motor is moving and taking data, (8) the decision step **384** to determine if scanning is to be continued, in which case the program returns to step **375** and if not, scanning is terminated at step **386**; and (9) the step **386** of terminating the scan. As shown in this diagram, the computer control moves the scanner from place to place taking measurements along a path to perform a scanning operation.

In FIG. **18**, there is shown a block diagram of the display **400** having a cathode ray tube **402**, a horizontal scan circuit **404**, a vertical scan circuit **406**, an electron gun **408**, a driver **410** and an intensity control **412**. As shown in this view, the horizontal scan control **404** periodically scans horizontally by applying a voltage to the deflection plates to move an electron beam from the gun **408** horizontally across the screen of the tube **402**. At a less rapid rate, the vertical control **406** changes the vertical voltage to deflect the electron beam and form a raster. During the formation of the raster, data is applied to the driver **410** from the computer (FIG. **10**) to modulate the voltage in the modulation control **412** to change the intensity corresponding to data. As shown in FIG. **19**, the face of the cathode ray tube **402** scans across with its data to form a plurality of bands **422** in which the existence of markers is shown by a different intensity of light on the screen so that DNA channels in the gel are shown as dark and light bands in a manner similar to that shown by gel electrophoresis. With this arrangement, the scanning rate may be set to discriminate against noise, particularly discriminating against the natural fluorescense of the glass in the gel sandwich **150** (FIGS. **6** and **7**). The screen display permits easy adjustment during measurements or data retrieval.

From the above summary, it can be understood that the sequencing techniques of this invention have several advantages, such as: (1) they take advantage of resolution over time, as opposed to space; (2) they are continuous; (3) they are automatic; (4) they are capable of sequencing or identifying markers in relatively long strands including strands of more than 100 bases; (5) they are relatively economical and easy to use; (6) they permit efficient focusing of a light sensor onto the DNA bands; (7) they provide an easy to observe display; (8) because the dyes have their emission spectra in the far red or near infrared light spectrum, small inexpensive far red or infrared laser diodes may be used; (9) the signal information is characterized by relatively low noise; and (10) they provide a simple protocol using universal termination of strands elongated by DNA polymerase.

While in the preferred embodiment, a single emission wavelength is used in the far red or near infrared region in each channel and for all of A, T, G and C terminated strands with the channel location identifying the terminating base type, multiple fluorescent markers can be used with the wavelength being used to identify the base type. In such an embodiment, an optical means detects a plurality of wavelengths and the computer correlates intensity data, corresponding lanes and corresponding wavelengths.

Although a preferred embodiment of the invention has been described with some particularity, many modifications and variations are possible in the preferred embodiment within the light of the above description. Accordingly, within the scope of the appended claims, the invention may be practiced other than as specifically described.

What is claimed is:

1. A method of DNA sequencing whereby DNA strands are used as templates to synthesize DNA fragments with limited synthesis, comprising the steps of using fluorescently marked A nucleotides to terminate synthesis at base type A, fluorescently marked G nucleotides to terminate synthesis at base type G, fluorescently marked C nucleotides to terminate synthesis at base type C, and fluorescently marked T nucleotides to terminate synthesis at base type T and analyzing the terminated DNA fragments so produced for the presence of fluorescent markers, thereby identifying the DNA sequence.

2. A process of DNA sequence analysis by chain elongation using terminators comprising reacting a template of DNA contained in an appropriate nucleotide, a primer, a polymerase, a first mixture of DNA nucleotides or their analogs, and a second mixture of terminators corresponding to the DNA nucleotides or their analogs, to produce fragments of DNA having terminators attached to a terminus introduced by chain termination events, wherein the terminators are fluorescently marked.

3. The process as set forth in claim **2** wherein the fragments of DNA having marked terminators attached to a terminus are analyzed for the presence of fluorescent markers, thereby identifying the DNA sequence.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,207,421 B1
DATED : March 27, 2001
INVENTOR(S) : Lyle Richard Middendorf and John A. Brumbaugh

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 28,
Line 27, change "contained in" to -- containing --.

Signed and Sealed this

Sixteenth Day of October, 2001

Attest:

Nicholas P. Godici

NICHOLAS P. GODICI

Attesting Officer

Acting Director of the United States Patent and Trademark Office